BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 17 July 2000 (17.07.00)	
International application No. PCT/GB99/03747	**Applicant's or agent's file reference** P023383WO
International filing date (day/month/year) 10 November 1999 (10.11.99)	**Priority date** (day/month/year) 10 November 1998 (10.11.98)
Applicant SMITH, Bryan, John	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

02 June 2000 (02.06.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

REC'D 12 FEB 2001
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PO23383WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03747	10/11/1999	10/11/1998
International Patent Classification (IPC) or national classification and IPC A61K47/48		
Applicant CELLTECH THERAPEUTICS LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/06/2000	08.02.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer G. Willière Telephone No. +49 89 2399 8548

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03747

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-43 as originally filed

Claims, No.:

1-13 as originally filed

Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	9-11
	No:	Claims	1-8,12 and 13
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-13
Industrial applicability (IA)	Yes:	Claims	1-13
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: WO 92 00763 A (AKZO N. V., NETH.) 23 January 1992 (1992-01-23)
   D2: WO 95 33492 A (HOUSTON BIOTECHNOLOGY) 14 December 1995 (1995-12-14)
   D3: YUKAWA N ET AL: 'Bispecific rabbit Fab'-bovine serum albumin conjugate used in hemagglutination immunoassay for β-microseminoprotein.' JOURNAL OF IMMUNOASSAY, (1997 AUG) 18 (3) 215-33.

2. D1 discloses IgM-HSA(-DTPA) conjugates (see page 6, third paragraph to page 7, second paragraph in combination with example 3), IgM Fab'-HSA(-DTPA) conjugates (see page 7, third and fourth paragraph in combination with example 3).

3. D2 refers to antibody-BSA-daumomycin conjugates (see example 5, page 30ff) as well as to Fab'-BSA-daumomycin conjugates (see example 11, page 37).

4. D3 discloses Fab'-BSA conjugates (see figure 1 at page 217).

5. It follows that the subject-matter of present claims 1 to 8, 12 and 13 is not novel with regard to the disclosure of D1 to D3 (article 33(2) PCT).

6. The subject-matter of the independent claims 9 to 11 appears to be novel (article 33(2) PCT). However the presence of an inventive step may only be regarded as to be present in combination with novel and inventive independent claims (article 33(3) PCT).

Re Item VII

Certain defects in the international application

1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art

disclosed in the documents D1 to D3 are not mentioned in the description, nor are these documents identified therein.

2. Independent claims 1 and 13 are not in the two-part form in accordance with Rule 6.3(b) PCT, which in the present case would be appropriate, with those features known in combination from the prior art (D1 to D3) being placed in a preamble (Rule 6.3(b)(i) PCT) and with the remaining features being included in a characterising part (Rule 6.3(b)(ii) PCT).

   Said claims should therefore be redrafted accordingly. If, however, the applicant is of the opinion that the two-part form would be inappropriate, then reasons therefor should be provided in the letter of reply. In addition, the applicant should ensure that it is clear from the description which features of the subject-matter of claims are known from documents D1 to D3 (see the PCT Guidelines PCT/GL/3 III, 2.3a).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 03747	10/11/1999	10/11/1998

Applicant

CELLTECH THERAPEUTICS LIMITED et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted]B 99/03747

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 00763 A (AKZO N. V., NETH.) 23 January 1992 (1992-01-23) page 2, paragraph 3 -page 3, paragraph 3 page 4, paragraph 2 example 2 ---	1-13
X	WO 95 33492 A (HOUSTON BIOTECHNOLOGY) 14 December 1995 (1995-12-14) figure 1 page 8, line 13-24 --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 February 2000	16/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Covone, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YUKAWA N ET AL: "Bispecific rabbit Fab'-bovine serum albumin conjugate used in hemagglutination immunoassay for beta-microseminoprotein." JOURNAL OF IMMUNOASSAY, (1997 AUG) 18 (3) 215-33. , XP000876816 abstract figure 1	1-13
A	WO 97 34631 A (UNIV TEXAS ;WARD ELIZABETH SALLY (US)) 25 September 1997 (1997-09-25) page 3, line 4-19 claim 1	1-13
A	YEH P ET AL: "Design of yeast-secreted albumin derivatives for human therapy: biological and antiviral properties of a serum albumin -CD4 genetic conjugate." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1992 MAR 1) 89 (5) 1904-8. , XP002130704 abstract page 1904, left-hand column, paragraph 2 -right-hand column, paragraph 2 page 1908, left-hand column, paragraphs 3,4	1-13
A	SYED S ET AL: "Potent antithrombin activity and delayed clearance from the circulation characterize recombinant hirudin genetically fused to albumin." BLOOD, (1997 MAY 1) 89 (9) 3243-52. , XP002130705 abstract page 3247, left-hand column, paragraph 3	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
P[redacted]B 99/03747

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9200763 A	23-01-1992	EP 0537222 A	21-04-1993
		FI 925804 A	21-12-1992
		HU 63343 A	30-08-1993
		NO 924762 A	29-12-1992
WO 9533492 A	14-12-1995	AU 2823495 A	04-01-1996
WO 9734631 A	25-09-1997	AU 1985097 A	10-10-1997
		CA 2249195 A	25-09-1997
		EP 0904107 A	31-03-1999

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MERCER, Christopher P.
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

PCT

12 FEB 2001

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	08.02.2001
Applicant's or agent's file reference PO23383WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03747	10/11/1999	10/11/1998

Applicant
CELLTECH THERAPEUTICS LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Gallego, A Tel.+49 89 2399-8102

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PO23383WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03747	10/11/1999	10/11/1998
International Patent Classification (IPC) or national classification and IPC A61K47/48		
Applicant CELLTECH THERAPEUTICS LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/06/2000	08.02.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer G. Willière Telephone No. +49 89 2399 8548

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03747

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-43 as originally filed

Claims, No.:

1-13 as originally filed

Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	9-11
	No:	Claims	1-8,12 and 13
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-13
Industrial applicability (IA)	Yes:	Claims	1-13
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: WO 92 00763 A (AKZO N. V., NETH.) 23 January 1992 (1992-01-23)
   D2: WO 95 33492 A (HOUSTON BIOTECHNOLOGY) 14 December 1995 (1995-12-14)
   D3: YUKAWA N ET AL: 'Bispecific rabbit Fab'-bovine serum albumin conjugate used in hemagglutination immunoassay for β-microseminoprotein.' JOURNAL OF IMMUNOASSAY, (1997 AUG) 18 (3) 215-33.

2. D1 discloses IgM-HSA(-DTPA) conjugates (see page 6, third paragraph to page 7, second paragraph in combination with example 3), IgM Fab'-HSA(-DTPA) conjugates (see page 7, third and fourth paragraph in combination with example 3).

3. D2 refers to antibody-BSA-daumomycin conjugates (see example 5, page 30ff) as well as to Fab'-BSA-daumomycin conjugates (see example 11, page 37).

4. D3 discloses Fab'-BSA conjugates (see figure 1 at page 217).

5. It follows that the subject-matter of present claims 1 to 8, 12 and 13 is not novel with regard to the disclosure of D1 to D3 (article 33(2) PCT).

6. The subject-matter of the independent claims 9 to 11 appears to be novel (article 33(2) PCT). However the presence of an inventive step may only be regarded as to be present in combination with novel and inventive independent claims (article 33(3) PCT).

Re Item VII

Certain defects in the international application

1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art

disclosed in the documents D1 to D3 are not mentioned in the description, nor are these documents identified therein.

2. Independent claims 1 and 13 are not in the two-part form in accordance with Rule 6.3(b) PCT, which in the present case would be appropriate, with those features known in combination from the prior art (D1 to D3) being placed in a preamble (Rule 6.3(b)(i) PCT) and with the remaining features being included in a characterising part (Rule 6.3(b)(ii) PCT).

Said claims should therefore be redrafted accordingly. If, however, the applicant is of the opinion that the two-part form would be inappropriate, then reasons therefor should be provided in the letter of reply. In addition, the applicant should ensure that it is clear from the description which features of the subject-matter of claims are known from documents D1 to D3 (see the PCT Guidelines PCT/GL/3 III, 2.3a).

DUE: 22-11-00

PATENT COOPERATION TREATY

PO23383WO

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
MERCER, Christopher P.
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	22.09.2000
REPLY DUE	**within 2 month(s)** from the above date of mailing

Applicant's or agent's file reference	International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PO23383WO	PCT/GB99/03747	10/11/1999	10/11/1998

International Patent Classification (IPC) or both national classification and IPC
A61K47/48

Applicant
CELLTECH THERAPEUTICS LIMITED et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☒	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 10/03/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
G. Willière

Formalities officer (incl. extension of time limits)
Gallego, A
Telephone No. +49 89 2399 8102

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-43 as originally filed

Claims, No.:

1-13 as originally filed

Drawings, sheets:

1/9-9/9 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-8,12 and 13: No
Inventive step (IS)	Claims	1-13: No
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: WO 92 00763 A (AKZO N. V., NETH.) 23 January 1992 (1992-01-23)
   D2: WO 95 33492 A (HOUSTON BIOTECHNOLOGY) 14 December 1995 (1995-12-14)
   D3: YUKAWA N ET AL: 'Bispecific rabbit Fab'-bovine serum albumin conjugate used in hemagglutination immunoassay for β-microseminoprotein.' JOURNAL OF IMMUNOASSAY, (1997 AUG) 18 (3) 215-33.

2. D1 discloses IgM-HSA(-DTPA) conjugates (see page 6, third paragraph to page 7, second paragraph in combination with example 3), IgM Fab'-HSA(-DTPA) conjugates (see page 7, third and fourth paragraph in combination with example 3).

3. D2 refers to antibody-BSA-daumomycin conjugates (see example 5, page 30ff) as well as to Fab'-BSA-daumomycin conjugates (see example 11, page 37).

4. D3 discloses Fab'-BSA conjugates (see figure 1 at page 217).

5. It follows that the subject-matter of present claims 1 to 8, 12 and 13 is not novel with regard to the disclosure of D1 to D3 (article 33(2) PCT).

6. The subject-matter of the independent claims 9 to 11 appears to be novel (article 33(2) PCT). However the presence of an inventive step may only be regarded as to be present in combination with novel and inventive independent claims (article 33(3) PCT).

7. After the Applicant succeeded in establishing novelty, he should demonstrate the presence of an inventive step.

   Non-obviousness of the claimed subject matter has to be demonstrated by

defining the difference between the claimed subject matter and the teaching of documents D1 to D3.

According to this difference the problem underlying the alleged invention has to be defined and the proposed solution (i.e. claimed subject-matter) has to be demonstrated to be non-obviously derivable from the closest prior art (D1 to D3). This can be shown by indication of advantageous and unexpected properties of hybrid protein of the present application.

8. Independent claims 1 and 13 are not in the two-part form in accordance with Rule 6.3(b) PCT, which in the present case would be appropriate, with those features known in combination from the prior art (D1 to D3) being placed in a preamble (Rule 6.3(b)(i) PCT) and with the remaining features being included in a characterising part (Rule 6.3(b)(ii) PCT).

Said claims should therefore be redrafted accordingly. If, however, the applicant is of the opinion that the two-part form would be inappropriate, then reasons therefor should be provided in the letter of reply. In addition, the applicant should ensure that it is clear from the description which features of the subject-matter of claims are known from documents D1 to D3 (see the PCT Guidelines PCT/GL/3 III, 2.3a).

9. The Applicant is requested to file amendments by way of replacement pages in the manner stipulated by Rule 66.8(a) PCT. In particular, fair copies of the amendments should be filed preferably in triplicate.

Moreover, the Applicant's attention is drawn to the fact that, as a consequence of Rule 66.8(a) PCT the examiner is not permitted to carry out any amendments under the PCT procedure, however minor these may be.

10. In order to facilitate the examination of the conformity of the amended application with the requirements of Article 34(2)(b) PCT, the applicant is requested to clearly identify the amendments carried out, no matter whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (see also Rule 66.8(a) PCT).

If the applicant regards it as appropriate these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed.

11. Any information the applicant may wish to submit concerning the subject-matter of the invention, for example further details of its advantages or of the problem it solves, and for which there is no basis in the application as filed, should be confined to the letter of reply rather than be incorporated into the application, Article 34(2)(b) PCT.

Re Item VII

Certain defects in the international application

1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 to D3 are not mentioned in the description, nor are these documents identified therein.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P023383WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 03747	10/11/1999	10/11/1998
Applicant		
CELLTECH THERAPEUTICS LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted] GB 99/03747

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 00763 A (AKZO N. V., NETH.) 23 January 1992 (1992-01-23) page 2, paragraph 3 -page 3, paragraph 3 page 4, paragraph 2 example 2 ---	1-13
X	WO 95 33492 A (HOUSTON BIOTECHNOLOGY) 14 December 1995 (1995-12-14) figure 1 page 8, line 13-24 --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 February 2000	16/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Covone, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YUKAWA N ET AL: "Bispecific rabbit Fab'-bovine serum albumin conjugate used in hemagglutination immunoassay for beta-microseminoprotein." JOURNAL OF IMMUNOASSAY, (1997 AUG) 18 (3) 215-33. , XP000876816 abstract figure 1 ---	1-13
A	WO 97 34631 A (UNIV TEXAS ;WARD ELIZABETH SALLY (US)) 25 September 1997 (1997-09-25) page 3, line 4-19 claim 1 ---	1-13
A	YEH P ET AL: "Design of yeast-secreted albumin derivatives for human therapy: biological and antiviral properties of a serum albumin -CD4 genetic conjugate." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1992 MAR 1) 89 (5) 1904-8. , XP002130704 abstract page 1904, left-hand column, paragraph 2 -right-hand column, paragraph 2 page 1908, left-hand column, paragraphs 3,4 ---	1-13
A	SYED S ET AL: "Potent antithrombin activity and delayed clearance from the circulation characterize recombinant hirudin genetically fused to albumin." BLOOD, (1997 MAY 1) 89 (9) 3243-52. , XP002130705 abstract page 3247, left-hand column, paragraph 3 -----	1-13

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
[redacted]GB 99/03747

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9200763 A	23-01-1992	EP 0537222 A	21-04-1993
		FI 925804 A	21-12-1992
		HU 63343 A	30-08-1993
		NO 924762 A	29-12-1992
WO 9533492 A	14-12-1995	AU 2823495 A	04-01-1996
WO 9734631 A	25-09-1997	AU 1985097 A	10-10-1997
		CA 2249195 A	25-09-1997
		EP 0904107 A	31-03-1999

PCT International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 47/48

A1

(11) International Publication Number: WO 00/27435

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/GB99/03747

(22) International Filing Date: 10 November 1999 (10.11.99)

(30) Priority Data:
9824632.5 10 November 1998 (10.11.98) GB

(71) Applicant *(for all designated States except US)*: CELLTECH THERAPEUTICS LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: SMITH, Bryan, John [GB/GB]; 20 Belmont Drive, Maidenhead, Berkshire SL6 6JZ (GB).

(74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTIBODY–SERUM PROTEIN HYBRIDS

(57) Abstract

Hybrid proteins are described which comprise one or more antigen–binding antibody fragments covalently linked to one or more serum carrier proteins. The hybrid proteins can bind antigens, have a long half–life *in vivo* and can be used in medicine for therapy and diagnosis.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

ANTIBODY-SERUM PROTEIN HYBRIDS

This invention relates to modified antibody fragments, to processes for their preparation and to their use in medicine.

Since antibodies show great specificity in their binding to other molecules, they are of benefit as therapeutic or diagnostic agents, or as reagents (for example, as affinity purification reagents or as catalytic enzymes). The advent of hybridoma technology and the generation of transgenic animals has allowed the production of large volumes of monoclonal antibodies, sufficient for therapeutic use. Most such antibodies have been generated for treatment of acute diseases, such as particular types of cancer. For the treatment of chronic and/or more common diseases, still larger amounts of antibody are likely to be required. An increased usage also brings an increased need for a cheaper therapeutic agent.

Monoclonal antibodies can be produced in cultured mammalian or insect cells, or in transgenic animals, but the ability of these technologies to supply a large market at a reasonable cost, is unproven so far. Again, fungi have been shown to be able to produce heterologous proteins [e.g. Sleep, D., *et al* (1991) Bio/Technology, 9, 183-187], but expression of whole immunoglobulin G (IgG1) in a fungus has been reported to occur only at low level (in *Saccharomyces cerevisiae*), or in shakeflask culture (in *Pichia pastoris*), so antibody production from fungi on an industrial scale is unproven. [Horwitz, A. H., *et al* (1988) Proc. Natl. Acad. Sci. USA, 85, 8678-8682; Ogunjimi, A.A. *et al* (1999) Biotechnology Lett. 21, 561-567]. Furthermore, glycosylation of the antibody would be expected to be unlike that from mammalian systems, and in mammals this may generate problems of immunogenicity and abnormal function (complement activation, binding to Fc receptors, transcytosis and prolongation of half-life through interaction with FcRn receptor) [Nose, M. and Wigzell, H. (1983) Proc. Natl.

Acad. Sci. USA, 80, 6632-6636; Tao, M.-H. and Morrison, S. L. (1989) J. Immunol. 143, 2595-2601; Wawrzynczak, E. J. *et al* (1992) Molec. Immunol. 29, 213-220; Kim, J.-K., *et al* (1994) Eur. J. Immunol. 24, 2429-2434].

Bacterial systems are not known to be capable of producing a whole, functioning antibody in a yield sufficient to give an economic process for large-scale manufacture, but they are a source of low-cost immunoglobulin fragments, such as Fab' [Better, M., *et al* (1988) Science, 240, 1041-1043]. Antibody fragments, however, may lack various of the functions of whole antibody. For example, Fab', $F(ab')_2$ or scFv lack the Fc domain that imparts a long lifetime *in vivo*: [Medesan, C. *et al* (1997) J. Immunol. 158, 2211-2217]. The half-life in circulation in mammals of Fab' or $F(ab')_2$ has been reported as being about 1% that of whole IgG [Waldmann, T. A. and Strober, W. (1969) Progr. Allergy, 13, 1-110.], and the β-phase half-life (the time taken for half of the molecules in circulation to be eliminated) of Fab' has been reported as being about 5% that of whole IgG [Chapman, A. P., *et al* (1999) Nature Biotechnology, 17, 780-783]. Thus, while they may be cheap to produce, these fragments are eliminated rapidly from circulation and can be of limited therapeutic use. This has led to attempts to prolong half-life of antibody fragments, for instance by modification of Fab' or $F(ab')_2$ *in vitro* by addition of one or more molecules of polyethylene glycol to each fragment molecule. International Patent Specification No. WO98/925971

In the present invention we have addressed the identifiable need for a means to economically produce an IgG fragment that can bind antigen and that has a long half-life *in vivo*. We have achieved this by employing a carrier protein to prolong the immunoglobulin in circulation.

Thus according to one aspect of the invention we provide a multi-component hybrid protein comprising one or more antigen-binding antibody

fragments covalently linked to one or more serum carrier proteins or fragments thereof.

A variety of proteins exist in plasma, circulating in the body with half-lives measured in days, for example, 5 days for thyroxine-binding protein or 2 days for transthyretin [Bartalena, L. and Robbins, J. (1993) Clinics in Lab. Med. 13, 583-598], or 65h in the second phase of turnover of iodinated α1-acid glycoprotein [Bree, F. *et al* (1986) Clin. Pharmacokin. 11, 336-342]. Again, data from Gitlin et al. [Gitlin, D., *et al* (1964) J. Clin. Invest. 10, 1938-1951] suggest that in pregnant women the half-life of α1-acid glycoprotein is 3.8 days, 12 days for transferrin and 2.5 days for fibrinogen.

Serum albumin is an abundant protein in both vascular and extravascular compartments [Peters, Jr., T. (1985) Adv. Prot. Chem. 37, 161-245]. The half-life of albumin in man, about 19 days [Peters,1985 *ibid*], is similar to that of IgG1 (about 21 days [Waldeman+Strober *ibid*], though it is less in other species - about 2 days in rats, for example [Peters, 1985 *ibid*]. Albumin does not possess the noted ability of antibodies to specifically bind ligands, particularly those of high molecular weight.

In the present invention we have produced a series of hybrid proteins that advantageously have the antigen-binding capabilities of an antibody fragment and the longevity of serum albumin *in vivo*. Additionally the hybrids may be especially suitable for use in expectant or nursing mothers since as with various other serum proteins, albumin is transported poorly across the placenta: labelled albumin injected into a mother appears with 5% or less specific activity in the foetus after 25 days [Gitlin, D. Kumate, J. Urrusti, J. and Morales, C. (1964) J. Clin. Invest. 10, 1938-1951]. This contrasts with IgG, which is transported efficiently across the placenta to the foetus. Similarly, albumin is not transported across the gut wall of neonates, whereas IgG is, by interaction with the FcRn receptor. Thus

advantageously a foetus or neonate would be exposed to minimal amounts of the hybrids according to the invention from maternal circulation or milk.

With regard to commercial production, recombinant albumin has been reported as being produced at several gm per litre of culture of yeast (*Pichia pastoris* or *Kluyveromyces lactis* [Barr, K. A., *et al* (1992) Pharm. Eng. 12, 48-51; Fleer, R., *et al* (1991) Bio/Technology 9, 968-975; Cregg, J. M., *et al* (1993) Bio/Technology 11, 905-910]). This level of expression makes industrial production of pharmaceutical grade protein economically feasible, as remarked by Fleer *et al* [Fleer, R., *et al* (1991) Bio/Technology 9, 968-975]. Similarly, transgenic mice have been found to express as much as 10 gm per litre of albumin in their milk [Hurwitz, D. R, *et al* (1994) Transgenic Res. 3,365-375].

Depending on the intended specific use and/or half-life required, the hybrid protein according to the invention may be in a number of different forms. For example, one protein according to the invention may comprise an antibody fragment covalently linked to two, three or more serum carrier proteins or fragments thereof each of which may be the same or different. In another example, a protein according to the invention may comprise two, three or more antibody fragments, which may be the same or different, each covalently linked to the same serum protein or a fragment thereof. In general, however, a preferred hybrid protein according to the invention comprises one, two or three antigen-binding antibody fragments covalently linked to one serum carrier protein or a fragment thereof.

Each antigen-binding antibody fragment component in the proteins according to the invention will in general comprise an antibody variable region domain containing one or more antigen binding sites.

The variable region domain may be of any size or amino acid composition

and will generally comprise at least one hypervariable amino acid sequence responsible for antigen binding embedded in a framework sequence. In general terms the variable (V) region domain may be any suitable arrangement of immunoglobulin heavy (V_H) and/or light (V_L) chain variable domains. Thus for example the V region domain may be monomeric and be a V_H or V_L domain where these are capable of independently binding antigen with acceptable affinity. Alternatively the V region domain may be dimeric and contain V_H-V_H, V_H-V_L, or V_L-V_L, dimers in which the V_H and V_L chains are non-covalently associated. Where desired, however, the chains may be covalently coupled either directly, for example via a disulphide bond between the two variable domains, or through a linker, for example a peptide linker, to form a single chain domain, e.g. scFv.

The variable region domain may be any naturally-occurring variable domain or an engineered version thereof. By engineered version is meant a variable region domain that has been created using recombinant DNA engineering techniques. Such engineered versions include those created for example from natural antibody variable regions by insertions, deletions or changes in or to the amino acid sequences of the natural antibodies. Particular examples of this type include those engineered variable region domains containing at least one CDR and optionally one or more framework amino acids from one antibody and the remainder of the variable region domain from a second antibody.

The variable region domain will in general be capable of selectively binding to an antigen. The antigen may be any cell-associated antigen, for example a cell surface antigen such as a T-cell, endothelial cell or tumour cell marker, or it may be an extracellular matrix antigen, an intracellular antigen or a soluble antigen. Particular examples of cell surface antigens include adhesion molecules, for example integrins such as β1 integrins, e.g. VLA-4,

E-selectin, P-selectin or L-selectin, CD2, CD3, CD4, CD5, CD7, CD8, CD11a, CD11b, CD18, CD19, CD20, CD23, CD25, CD33, CD38, CD40, CD45, CDW52, CD69, carcinoembryonic antigen (CEA), human milk fat globulin (HMFG1 and 2), MHC Class I and MHC Class II antigens, and VEGF, and where appropriate, receptors thereof. Soluble antigens include interleukins such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 or IL-12, viral antigens, for example respiratory syncytial virus or cytomegalovirus antigens, immunoglobulins, such as IgE, interferons such as interferon-α, interferon-β or interferon-γ, tumour necrosis factor-α, tumour necrosis factor-β, colony stimulating factors such as G-CSF or GM-CSF, and platelet derived growth factors such as PDGF-α, and PDGF-β and where appropriate receptors thereof.

In practice it is generally preferable that the variable region domain is covalently attached at a C-terminal amino acid to at least one other antibody domain or a fragment thereof. Thus, for example where a V_H domain is present in the variable region domain this may be linked to an immunoglobulin C_H1 domain or a fragment thereof. Similarly a V_L domain may be linked to a C_K domain or a fragment thereof. In this way for example the fragment according to the invention may be a Fab fragment wherein the antigen binding domain contains associated V_H and V_L domains covalently linked at their C-termini to a CH1 and C_K domain respectively. The CH1 domain may be extended with further amino acids, for example to provide a hinge region domain as found in a Fab' fragment, or to provide further domains, such as antibody CH2 and CH3 domains.

Each serum carrier protein component in the hybrid proteins according to the invention may be a naturally occurring serum carrier protein or a fragment thereof. Particular examples include thyroxine-binding protein,

transthyretin, α1-acid glycoprotein, transferrin, fibrinogen and, especially, albumin, together with fragments thereof. The carrier proteins will in particular be of human origin. Where desired each may have one or more additional or different amino acids to the naturally occurring sequence providing always that the resulting sequence is functionally equivalent with respect to half-life. Fragments include any smaller part of the parent protein that retains the carrier function of the mature sequence.

The antibody and carrier protein components in the hybrid proteins according to the invention may be directly or indirectly covalently linked. Indirect covalent linkage is intended to mean that an amino-acid in an antibody fragment is attached to an amino-acid in a carrier protein through an intervening chemical sequence, for example a bridging group. Particular bridging groups include for example aliphatic, including peptide, chains as more particularly described hereinafter. Direct covalent linkage is intended to mean that an amino acid in an antibody fragment is immediately attached to an amino acid in a carrier protein without an intervening bridging group. Particular examples include disulphide (-S-S-] and amide [-CONH-] linkages, for example when a cysteine residue in one component is linked to a cysteine residue in another through the thiol group in each, and when the C-terminal acid function of one component is linked to the N-terminal amine of the other.

Particular bridging groups useful to indirectly link an antibody to a carrier protein include optionally substituted aliphatic, cycloaliphatic, heteroaliphatic, heterocycloaliphatic, aromatic and heteroaromatic groups. Particular groups include optionally substituted straight or branched C_{1-20}alkylene; C_{2-20}alkenylene or C_{2-20}alkynylene chains optionally interrupted and/or terminally substituted by one or more -O- or -S- atoms, or -N(R^1)- [where R^1 is a hydrogen atom or a C_{1-6}alkyl group], -N(R^1)CO-, -CON(R^1)-,

$-N(R^1)SO_2-$, $-SO_2N(R^1)-$, -C(O)-, -C(O)O-, -OC(O)-, -S(O)-, $-S(O)_2-$, aromatic, e.g. phenyl, hereroaromatic, e.g. pyridyl, or cycloalkyl, e.g. cyclohexyl groups. Such chains include for example optionally substituted straight or branched C_{1-10}alkylene chains such as optionally substituted butylene, pentylene, hexylene or heptylene chains, single amino acid residues and peptide chains, for example containing two to twenty amino acids, which may be the same or different, e.g. polyglycine chains such as $(Gly)_n$ where n is an integer from two to ten. Optional substituents which may be present on any of the above chains include one or more halogen atoms, e.g. fluorine, chlorine, bromine or iodine atoms, or C_{1-6}alkyl, C_{1-6}alkoxy, C_{1-6}haloalkyl, C_{1-6}haloalkoxy, -OH or $-N(R^1)(R^2)$ [where R^2 is as defined for R^1] groups.

Where a bridging group indirectly links an antibody to a carrier protein the linkage may be to the side chain of any suitable amino acid, for example a lysine, arginine, serine, aspartic acid, glutamic acid or cysteine residue, located in the antibody or carrier protein. At each point of attachment the residue of a reactive group may be present. For example, where the bridging group is linked to a cysteine residue in the antibody or carried protein, the residue of a thiol-selective reactive group such as a maleimide group or the like may be incorporated as part of the attachment.

Where desired, the hybrid protein according to the invention may have one or more effector or reporter molecules attached to it and the invention extends to such modified proteins. The effector or reporter molecules may be attached to the antibody fragment and/or the carrier protein through any available amino acid side-chain or terminal amino acid functional group located in either component, for example any free amino, imino, hydroxyl or carboxyl group. The linkage may be direct or indirect, through spacing or

bridging groups, as just described above, for linking the antibody and carrier protein components. Alternatively the reporter/effector may be attached to the linking moiety

Effector molecules include, for example, antineoplastic agents, toxins (such as enzymatically active toxins of bacterial or plant origin and fragments thereof e.g. ricin and fragments thereof) biologically active proteins, for example enzymes, nucleic acids and fragments thereof, e.g. DNA, RNA and fragments thereof, natural or synthetic polymers such as polysaccharides or polyalkylene polymers such as poly(ethylene glycol), radionuclides, particularly radioiodide, and chelated metals. Suitable reporter groups include chelated metals, fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy.

Particular antineoplastic agents include cytotoxic and cytostatic agents, for example alkylating agents, such as nitrogen mustards (e.g. chlorambucil, melphalan, mechlorethamine, cyclophosphamide, or uracil mustard) and derivatives thereof, triethylenephosphoramide, triethylenethiophosphoramide, busulphan, or cisplatin; antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid, antibiotics, such as bleomycins (e.g. bleomycin sulphate), doxorubicin, daunorubicin, mitomycins (e.g. mitomycin C), actinomycins (e.g. dactinomycin) plicamycin, calichaemicin and derivatives thereof, or esperamicin and derivatives thereof; mitotic inhibitors, such as etoposide, vincristine or vinblastine and derivatives thereof; alkaloids, such as ellipticine; polyols such as taxicin-I or taxicin-II; hormones, such as androgens (e.g. dromostanolone or testolactone), progestins (e.g. megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. dimethylstilbestrol diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen); anthraquinones, such as mitoxantrone, ureas, such as hydroxyurea; hydrazines, such as

procarbazine; or imidazoles, such as dacarbazine.

Particularly useful effector groups are calichaemicin and derivatives thereof (see for example South African Patent Specifications Nos. 85/8794, 88/8127 and 90/2839).

Chelated metals include chelates of di-or tripositive metals having a coordination number from 2 to 8 inclusive. Particular examples of such metals include technetium (Tc), rhenium (Re), cobalt (Co), copper (Cu), gold (Au), silver (Ag), lead (Pb), bismuth (Bi), indium (In), gallium (Ga), yttrium (Y), terbium (Tb), gadolinium (Gd), and scandium (Sc). In general the metal is preferably a radionuclide. Particular radionuclides include ^{99m}Tc, ^{186}Re, ^{188}Re, ^{58}Co, ^{60}Co, ^{67}Cu, ^{195}Au, ^{199}Au, ^{110}Ag, ^{203}Pb, ^{206}Bi, ^{207}Bi, ^{111}In, ^{67}Ga, ^{68}Ga, ^{88}Y, ^{90}Y, ^{160}Tb, ^{153}Gd and ^{47}Sc.

The chelated metal may be for example one of the above types of metal chelated with any suitable polydentate chelating agent, for example acyclic or cyclic polyamines, polyethers, (e.g. crown ethers and derivatives thereof); polyamides; porphyrins; and carbocyclic derivatives.

In general, the type of chelating agent will depend on the metal in use. One particularly useful group of chelating agents in conjugates according to the invention, however, are acyclic and cyclic polyamines, especially polyaminocarboxylic acids, for example diethylenetriaminepentaacetic acid and derivatives thereof, and macrocyclic amines, e.g. cyclic tri-aza and tetra-aza derivatives (for example as described in International Patent Specification No. WO 92/22583); and polyamides, especially desferrioxamine and derivatives thereof.

Particularly useful hybrid proteins according to the invention include one or

more antigen-binding antibody fragments covalently linked to one or more albumin molecules or fragments thereof.

Particular hybrids of this type include those wherein one antigen-binding antibody fragment is covalently linked to an albumin molecule or a fragment thereof. In these hybrids, and in general in the proteins according to the invention, each antigen-binding antibody fragment is preferably a monovalent Fab' fragment, optionally containing one or more additional amino acids attached to the C-terminus of the CH1 domain and is especially a Fab' fragment. Particularly useful Fab' fragments include those wherein the hinge domain contains a single cysteine residue. Fragments of albumin include one or more of domains I, II and/or III or subdomains thereof [see for example Peters, T. in "All about Albumin", Academic press, London (1996)].

Especially useful antibody-albumin hybrids according to the invention include those in which each protein component is directly linked through the C-terminal amino acid of the antibody to the N-terminal amino acid of the albumin. Where desired, one or more, e.g. up to around 100, additional amino acids may be inserted between the C- and N-termini to form a spacing group.

Another particularly useful class of antibody-albumin hybrids according to the invention is that wherein each protein component is indirectly linked between the thiol groups of a cysteine residue present in the antibody and another in the albumin. The indirect linkage may be achieved by a bridging molecule as described above. Particularly useful groups include non-cleavable linker groups, especially optionally substituted straight or branched C_{1-10} alkylene chains.

In this class of hybrids the antibody is preferably a Fab' fragment optionally containing one or more additional amino acids attached to the C-terminal of the CH1 domain. Especially useful fragments include Fab' fragments. The cysteine residue to which the spacing or bridging molecule is attached is preferably located in the CH1 domain of the Fab or, especially, is located in any C-terminal extension of the CH1 domain of the Fab, for example in the hinge domain of a Fab'.

The albumin in this class of hybrids may be in particular mature human serum albumin or a fragment thereof. In this instance, the bridging molecule may be attached to the cysteine residue at position 34 of the albumin. Advantageously, to avoid undesirable homodimer formation [see the Examples below] the bridging molecule may be from around 10Å to around 20Å in length, for example around 16Å. Suitable bridging molecules in this length range may be easily determined from published sources, for example manufacturers' catalogues [see below]. Particularly useful bridging molecules include optionally substituted hexylene chains. Where each end of the bridging molecule is attached to the cysteine residue this may be through a disulphide bond or, in particular, a sulphur-carbon bond. Where the linkage is a sulphur-carbon bond, the residue of a thiol-selective reactive group, such as a maleimide, may be present as part of each end of the spacing or bridging group.

The hybrid proteins according to the invention may be useful in the detection or treatment of a number of diseases or disorders. Such diseases or disorders may include those described under the general headings of infectious disease, e.g. viral infection; inflammatory disease/autoimmunity e.g. rheumatoid arthritis, osteoarthritis, inflammatory bowel disease; cancer; allergic/atopic disease e.g. asthma, eczema; congenital disease, e.g. cystic fibrosis, sickle cell anaemia; dermatologic disease, e.g. psoriasis; neurologic disease, e.g. multiple sclerosis; transplants e.g. organ transplant rejection,

graft-versus-host disease; and metabolic/idiopathic disease e.g. diabetes.

The hybrid protein according to the invention may be formulated for use in therapy and/or diagnosis and according to a further aspect of the invention we provide a pharmaceutical composition comprising a multi-component hybrid protein comprising one or more antigen-binding antibody fragments covalently linked to one or more serum carrier proteins or fragments thereof, together with one or more pharmaceutically acceptable excipients, diluents or carriers.

As explained above, the hybrid protein in this aspect of the invention may be optionally linked to one or more effector or reporter groups.

The pharmaceutical composition may take any suitable form for administration, e.g. for oral, buccal, parenteral, nasal, topical or rectal administration, or a form suitable for administration ny inhalation or insufflation, and preferably is in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the composition is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents such as suspending, preservative, stabilising, antioxidant and/or dispersing agents.

Alternatively, the composition may be in dry form, for reconstitution before use with an appropriate sterile liquid.

If the composition is suitable for oral administration the formulation may contain, in addition to the active ingredient, additives such as: starch e.g. potato, maize or wheat starch or cellulose or starch derivatives such as microcrystalline cellulose; silica; various sugars such as lactose; magnesium carbonate and/or calcium phosphate. It is desirable that, if the

formulation is for oral administration it will be well tolerated by the patient's digestive system. To this end, it may be desirable to include in the formulation mucus formers and resins. It may also be desirable to improve tolerance by formulating the antibody in a capsule which is insoluble in the gastric juices. It may also be preferable to include the antibody or composition in a controlled release formulation.

If the composition is suitable for rectal administration the formulation may contain a binding and/or lubricating agent; for example polymeric glycols, gelatins, cocoa-butter or other vegetable waxes or fats.

Therapeutic and diagnostic uses of hybrid proteins according to the invention typically comprise administering an effective amount of the protein to a human subject. The exact amount to be administered will vary according to the intended use of the protein and on the age, sex and condition of the patient but may typically be varied from about 0.1mg to 1000mg for example from about 1mg to 500mg. The protein may be administered as a single dose or in a continuous manner over a period of time. Doses may be repeated as appropriate. Typical doses may be for example between 0.1-50 mg/kg body weight per single therapeutic dose, particularly between 0.1-20 mg/kg body weight for a single therapeutic dose.

The hybrid proteins according to the invention may be prepared by standard chemical, enzymatic and/or recombinant DNA procedures.

Thus for example the hybrid protein may be prepared by the use of recombinant DNA techniques involving the manipulation and re-expression of DNA encoding antibody variable and/or constant regions and a desired carrier protein or a fragment thereof. Such DNA is known and/or is readily available from DNA libraries including for example phage-antibody libraries

[see Chiswell, D J and McCafferty, J. Tibtech. 10, 80-84 (1992)] or where desired can be synthesised. Standard molecular biology and/or chemistry procedures may be used to sequence and manipulate the DNA, for example, to introduce codons to create cysteine residues, to modify, add or delete other amino acids or domains in the antibody and/or carrier protein as desired.

From here, one or more replicable expression vectors containing the DNA may be prepared and used to transform an appropriate cell line, e.g. a non-producing myeloma cell line, such as a mouse NSO line or a bacterial, e.g. *E.coli* line, or, especially, a fungal line, such as a yeast line, e.g. members of the genera *Pichia*, *Saccharomyces*, or *Kluyveromyces,* in which production of the antibody will occur. In order to obtain efficient transcription and translation, the DNA sequence in each vector should include appropriate regulatory sequences, particularly a promoter and leader sequence operably linked to the variable domain sequence. Particular methods for producing recombinant proteins in this way are generally well known and routinely used. For example, basic molecular biology procedures are described by Maniatis *et al* [Molecular Cloning, Cold Spring Harbor Laboratory, New York, 1989]; DNA sequencing can be performed as described in Sanger *et al* [PNAS 74, 5463, (1977)] and the Amersham International plc sequencing handbook; and site directed mutagenesis can be carried out according to the method of Kramer *et al* [Nucl. Acids Res. 12, 9441, (1984)] and the Anglian Biotechnology Ltd handbook. Additionally, there are numerous publications, including patent specifications, detailing techniques suitable for the preparation of proteins by manipulation of DNA, creation of expression vectors and transformation of appropriate cells for example as described in International Patent Specification No. WO86/01533 and European Patent Specification No. 392745.

Chemical synthesis of the hybrid proteins according to the invention may be

achieved by coupling appropriately functionalised antibody, carrier protein and, where appropriate, bridging groups in a predetermined order. Standard chemical coupling techniques may be employed utilising starting materials containing one or more reactive functional groups such as thiols, acids, thioacids, anhydrides, acid halides, esters, imides, aldehydes, ketones, imines and amines. The starting antibody and carrier protein may be readily obtained from natural sources and/or by recombinant DNA techniques as described previously. Suitable bridging groups, for example in the 10Å-20Å length range as described above, are either commercially available [see for example Pierce & Warriner (UK) Ltd., Chester, UK] or may be obtained by simple functionalisation of known readily available chemical using convention chemistry.

Thus in one general approach, a homo- or heteropolyfunctional e.g. bi- or trifunctional bridging group, may first be coupled to either the antibody or carrier protein and the resulting product coupled as necessary to the remaining component(s) to provide the hybrid protein of the invention. The coupling reactions may be performed using standard conditions for reactions of this type. Thus for example the reaction may be performed in a solvent, for example an organic solvent, an aqueous-organic solvent or, especially, an aqueous solvent at or around ambient temperature up to around 70°C. Preferably, to avoid unwanted polymerisation in the first coupling reaction the homo- or heteropolyfunctional bridging group is employed in excess concentration relative to the antibody or carrier protein. Similarly in the second coupling reaction, the antibody or carrier protein is preferably employed in excess concentration to the product of the first coupling reaction. Illustrative reactions are described in detail in the Examples hereinafter for the preparation of proteins according to the invention and these may be readily adapted using different starting materials to provide other compounds of the invention.

The following Examples illustrate the invention. In these reference is made to various figures which are:

Figure 1.

SDS PAGE of RSA-Fab' conjugate.

The RSA-Fab' conjugate was run under both reducing and non-reducing conditions. Approximate apparent molecular weights were estimated by comparison with standard proteins run on the same gel under reducing conditions. The molar ratios were determined by N-terminal sequencing of bands blotted from gels onto PVDF membrane.

Figure 2.

Pharmacokinetics of RSA-Fab' conjugate or controls in the rat.

Proteins were ^{125}I-labelled prior to injection into 6 rats. Radioactivity present in blood sample taken at intervals was quantified by gammacounting. Results of analysis by Winnonlin are shown. The units for the plasma half-life in α and β phases ($t_{1/2}\alpha$ and $t_{1/2}\beta$, respectively) are hours (h). The areas under the plasma concentration time curves (AUC, 0-∞) are in the units h*%dose. Data plotted as RSA-Fab' conjugate (correction factor) have been corrected from data for RSA-Fab' conjugate to allow for instability of labeled protein, as decribed in the text.

Figure 3.

Pharmacokinetics of RSA-Fab' or Fab'-cys in the rat (assayed by ELISA or cytokine neutralisation).

Unlabeled proteins were injected into 2 rats, and subsequently quantified in samples of plasma by ELISA. In the case of the conjugate the ELISA employed binding of ligand (TNF) and of anti-albumin antibody, and in the case of the Fab'-cys control it employed binding of TNF and of anti-kappa L chain, as described in the text. The units for the plasma half-life in α and β

phases ($t_{1/2}\alpha$ and $t_{1/2}\beta$, respectively) are hours (h). Alternatively, samples were assayed by neutralisation of TNF activityin the L929 assay. The areas under the plasma concentration time curves (AUC, 0-∞) are in the units h*%μg/ml. Curves for RSA-Fab' overlay each other, and curves for Fab'-cys do likewise. Error bars = s.e.m.

Figure 4.

Construction of pPIC(scFv-HSA)

Flow chart of construction method.

Figure 5.

Reducing SDS PAGE of HSA-scFv fusion proteins.

A sample of each fusion protein before and after Blue sepharose chromatography was run under reducing conditions, together with standard molecular weight markers and standard RSA on the same gel.

Figure 6.

Pharmacokinetics of HSA-Fab' fusion proteins or HSA in the rat.

Proteins were ^{125}I-labelled prior to injection into 6 rats. Radioactivity present in blood sample taken at intervals was quantified by gammacounting. Results of analysis by Winnonlin are shown. The units for the plasma half-life in α and β phases ($t_{1/2}\alpha$ and $t_{1/2}\beta$, respectively) are hours (h). The areas under the plasma concentration time curves (AUC, 0-∞) are in the units h*%dose.

Figure 7.

Structure of trimaleimide crosslinking agent.

This reagent was used in generation of RSA-F(ab')$_2$ conjugate.

Figure 8.

Reducing SDS PAGE of HSA-F(ab')$_2$ conjugate.

Purified sample and standard proteins were run on the same gel under reducing conditions. The molar ratios were determined by N-terminal sequencing of bands blotted from gels onto PVDF membrane.

Figure 9.

Scatchard plots of binding of RSA-F(ab')$_2$ or F(ab')$_2$ control to cell membranes.

2 values for K_D were calculated for each of the (biphasic) curves, as shown. Each phase of each curve is shown as a straight line

Example 1:

Fab'-albumin conjugate.

Methods

Preparation of anti-TNF Fab'

Recombinant anti-TNF Fab' was produced in *E. coli,* and prepared from the periplasm by the methods described in International Patent Specification No. WO98/25971.

Conjugation of the anti-TNF Fab' with rat serum albumin

Rat serum albumin (RSA, fraction V, Sigma, code no. A-6272) was dissolved to 6.7mg/ml (0.1mM) in sodium acetate, 0.1M, pH5.9. Dithiothreitol solution (100mM in the same acetate buffer) was added to give a final dithiothreitol concentration of 0.3M, so giving a 3-fold molar excess over RSA. The mixture was incubated at 37°C for 40 min. The mixture was then subjected to chromatography on Sephadex G25M using a PD10 column (Pharmacia, code no. 17-0851-01, used as per manufacturer's instructions), thus removing dithiothreitol and exchanging the buffer for sodium phosphate, 0.1M, pH6, 2mM ethylenediamine-

tetraacetate (EDTA). The RSA concentration at that stage was 70μM.

1,6 bismaleimidohexane (BMH, Pierce, code no. 22330) was dissolved to 7.736mg/ml (28 mM) in dimethylformamide. The BMH solution was added to the reduced RSA solution to give a 21-fold molar excess of BMH over RSA. The mixture was incubated at 21°C for 100min, then subjected to chromatography on G25M in a buffer of sodium phosphate, 0.1M, pH6, 2mM EDTA. The concentration of derivatised RSA was 46μM at that stage.

The solutions of derivatised RSA and the anti-TNF Fab' (187μM) in sodium phosphate, 0.1M, pH6, 2mM EDTA were mixed to give a molar ratio, RSA:Fab':1:1.3 (which, corrected for derivatisation of RSA and reduction of Fab' thiol gave a ratio, derivatised RSA:reduced Fab':1:1.4). The mixture was incubated at 21°C for 2h, although reaction was essentially complete within 1h. The mixture was then stored at 4°C until subjected to purification procedures.

Conjugation of Fab' with cysteine

The method for preparation of control molecule, anti-TNF Fab' covalently linked by BMH via thiols to cysteine (instead of RSA) was essentially the same as for preparation of conjugate (see above). 20μM Fab' was reacted at 21°C for 95min with a 40-fold molar excess of BMH (added as a solution in dimethylformamide). After chromatography on G25M (to remove BMH) the derivatised Fab' was reacted with cysteine (Sigma, code no. C-4820) at a molar ratio Fab':cysteine free thiol:1:4.5. After reaction at 21°C for 160 min, the sample was stored at 4°C prior to purification of the Fab'-cys product.

Purification of the conjugate

The reaction mixture was first subjected to chromatography on Gammabind plus (Pharmacia), a matrix that has affinity for Fab'. A 5.3ml column was equilibrated in a buffer of sodium phosphate, 0.1M, pH6, 2mM EDTA at a flow rate of 2ml/min. All chromatography was at a temperature of 21°C. The sample was applied to the column at a flow rate of 1ml/min, and the column then washed in the same buffer (sodium phosphate, 0.1M, pH6, 2mM EDTA) until the baseline was restored. Adsorbed protein was eluted by application of a buffer of acetic acid, 0.5M, made to pH3 by addition of sodium hydroxide. The whole eluent was collected in fractions and each fraction analysed by SDS PAGE (using both reducing and non-reducing conditions). As expected of this affinity matrix, unconjugated Fab' eluted in the pH3 buffer, whereas the unconjugated RSA did not bind to the matrix at all, and emerged in the flow-through during sample loading. Conjugation of a single Fab' to one RSA molecule clearly affected its binding to the protein G on the matrix, for the conjugate emerged in the flow-through, just slightly later than (and overlapping with) the unconjugated RSA. The fraction containing the conjugate was subjected once more to chromatography on Gammabind plus (as above), in order to separate more RSA from it. The fractions containing conjugate (and some traces of RSA) were concentrated in a stirred cell (Amicon, 10 kDa nominal molecular weight cut-off membrane).

The contaminating RSA was removed from the preparation by gel permeation chromatography on a GF250 HPLC column of size 2x23cm (using a Hewlett Packard 1090 HPLC). The column was equilibrated and eluted in a buffer of sodium phosphate, 0.2M, pH7, at a flow rate of 3 mL/min, at 21°C. The sample of concentrated conjugate (and RSA) was chromatographed and elution monitored at 280 and 220nm. Fractions corresponding to observed peaks were collected and analysed by SDS

PAGE. Those fractions containing the conjugate (which was completely resolved from the later-eluting RSA) were pooled and concentrated in a stirred cell (Amicon, 10 kDa nominal molecular weight cut-off membrane). The solution was stored at 4°C, with sodium azide added to 0.05% (w/v), to act as a preservative.

Purification of the Fab'-cys

The reaction mixture containing Fab'-cys product was diluted 5-fold in a buffer of sodium acetate, 50mM, pH4.5, then loaded onto a Mono S column (of 1mL volume), using an FPLC (Pharmacia) apparatus. Adsorbed proteins were then eluted in a gradient of 0 to 250mM sodium chloride in sodium acetate, 50mM, pH4.5. Elution (1ml/min at 21°C) was monitored by absorption at 280nm. Collected peaks were analysed by SDS PAGE.

Radiolabelling of proteins

Proteins were labeled at the ε-amino groups of lysyl residues, using ^{125}I-labeled Bolton and Hunter reagent (Amersham International, code no. IM5861). Proteins were dissolved or diluted in a buffer of borate, to give a final borate concentration of 0.1M, pH8. A solution (of between 300 and 370μL) containing 300μg protein was then mixed with 20μL Bolton and Hunter solution in propan-2-ol (containing 9 MBq of ^{125}I). The mixture was incubated at 21°C for 15min, then the reaction was quenched by addition of 60μL solution of glycine, 1M, in borate, 0.1M, pH8.5. After approximately 5 min reaction at 21°C, the reaction mixture was chromatographed on Sephadex G25M using a PD10 column (Pharmacia, code no. 17-0851-01, used as per manufacturer's instructions). In doing so, the buffer was exchanged for phosphate buffered saline. The specific activity of each preparation was calculated from estimates of protein concentration (see Analytical Procedures) and of radioactivity, and were typically in the range

0.45 to 0.54μCi/μg. The radiolabeled samples were used directly after labeling.

Analytical procedures

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) utilised precast SDS gels (Novex), 1mm thick and of acrylamide concentration 4 to 20%, run as per manufacturer's instructions. Gels were stained by soaking for 1h in Coomassie BBG in perchloric acid (Sigma, code no. B-8772), followed by washing in water. Various molecular weight standards were used in order to derive approximate molecular weights (apparent) for sample proteins. These standards were Mark 12 and Seeblue unstained and prestained markers, respectively (Novex). For western blots, SDS PAGE was followed by blotting to polyvinylidene difluoride membrane (Millipore), with detection of the Fd fragment of the Fab' by sheep anti-human IgG(Fd) IgG fraction (The Binding Site, code no. PC075) followed by peroxidase-affinipure $F(ab')_2$ fragment of rabbit anti-sheep IgG, Fc fragment (Immunoresearch, code no. 313036046) and visualisation by use of chemiluminescence (ECL; Amersham International). For autoradiography, the SDS PAGE was followed by exposure of the gel to photographic film (Hyperfilm MP; Amersham International). For imaging of radiolabeled samples on gels (and subsequent quantification) the gels were exposed to high resolution or general purpose screens and processed in the Canberra Packard Cyclone system, using Optiquant software.

Quantification of protein solutions was by absorption at wavelength 280nm in a 1cm cell, using absorption coefficients (for 1mg/mL solution in a 1cm cell) of 1.43 for Fab' or $F(ab')_2$, and of 0.58 for RSA. A coefficient of 1.0 for RSA-Fab' conjugate was calculated from those of RSA and Fab', weighted in accordance with the constituent masses of the two components.

The concentration of free thiol in a protein solution was measured by adding 1/9 volume of 4,4'-dithiodipyridine (5mM, final concentration therefore was 0.5mM) in phosphate buffered saline. After 10min at 21°C, the absorbency at 324nm was measured in a 1cm cell. The absorbency of a buffer-only blank sample was subtracted from this value, and this figure multiplied by 56.1167 to give the result in μM thiol (this being further corrected for any dilution of the original sample).

N-terminal protein sequencing was performed as per manufacturer's instructions on a model 470A protein sequencer with on-line 120A HPLC and 900A data analysis system (Applied Biosystems). Protein in solution was adsorbed to polyvinylidene difluoride membrane in a Prosorb device (Applied Biosystems). Proteins from SDS PAGE were blotted to polyvinylidene difluoride membrane (Immobilon PSQ, Millipore), and protein bands detected by staining by 0.1% (w/v) Ponceau S (Sigma, code no. P-3504) in 1% (v/v) acetic acid for 1 min, then destaining the background in water. Bands were excised and sequenced directly.

Surface plasmon resonance study of interactions with ligand was performed on a BIACORE 2000 (Biacore AB), as per manufacturer's recommendations. Sensor chip surface coated by goat anti-human $F(ab')_2$ antibody (Jackson ImmunoResearch Lab. Inc.), which binds to the light chain, was used to bind conjugate, Fab' or IgG, whose binding to ligand (TNF) from solution was then measurable.

ELISA's were performed as follows below, with steps interspersed by washing in 0.1% Tween 20 in phosphate buffered saline. Microtitre plate wells were coated with cytokine antigen. Non-specific binding sites were then blocked by incubation of the wells with a 5% (w/v) solution of dried skimmed milk ("Marvel", Premier Beverages, UK) in phosphate buffered

saline, for 1h. Samples or standards were diluted in bovine serum albumin, 1% (w/v) in phosphate buffered saline, and then incubated in the wells for 1h at 21°C. Quantification was by absorption at 630nm, generated from 3,2'5,5' tetramethyl benzidine (120μM in 10mM acetate, pH6) as a result of the activity of peroxidase, following either of :

(i) For detection of albumin-Fab' conjugate, incubation of each well with rabbit anti-rat albumin (Cappel, product no. 55711, diluted 1 in 4000 in 1% bovine serum albumin in phosphate buffered saline), followed by goat anti-rabbit immunoglobulin Fc-peroxidase conjugate (Jackson, product no. 111-036-046, diluted 1 in 5000 in 1% (w/v) bovine serum albumin in phosphate buffered saline).

(ii) For detection of Fab', incubation in goat anti-human kappa light chain (Southern Biotechnology Associates, Inc., product no. 2060-01, diluted 1 in 5000 in 1% bovine serum albumin in phosphate buffered saline), followed by donkey anti-goat immunoglobulin (H+L)-peroxidase conjugate (Jackson, product no. 705-035-147, diluted 1 in 5000 in 1% (w/v) bovine serum albumin in phosphate buffered saline).

Assay format (i) gave a linear response in the range 40-500ng/ml of conjugate, and assay format (ii) gave a linear response in the range 5-100ng/ml of Fab'

To assay the neutralisation of TNF activity, a monolayer of mouse L929 cells was grown in normal RPMI 1640 plus glutamine and 10% (v/v) foetal calf serum. TNF added in presence of actinomycin D (1%, w/v) with or without sample/anti-TNF. Cell death caused by TNF was monitored by MTT assay: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, code no. M-2128) was added to a final concentration of 50μg/ml in the medium of treated cells and incubated at 37°C for 4h. Reaction was

stopped and the brown colour produced by live cells was solubilised by a solution of SDS, 20% (w/v) in 50% dimethylformamide in water, at pH4.7 (by addition of 50% acetic acid, 1M, in hydrochloric acid, 1M) and then quantified by measuring absorbance at 570nm. Having subtracted the absorbance at 630nm, the degree of cell survival (and so the concentration of active TNF present) could be assessed by comparison with samples of cells treated by standard amounts of TNF in the absence of TNF-neutralising activity.

Incubation of protein in plasma or blood *in vitro*

Rat plasma and blood (heparinised) were prepared fresh. To 100µl was added 12µl of ^{125}I-labeled protein solution. Incubation was at 37°C in a capped 0.5ml tube, with 0.5 and 2µL samples withdrawn at intervals far SDS PAGE and autoradiography.

Unlabeled conjugate or Fab'-cys (2µlof 0.9 mg/ml solution) was mixed with 100µl fresh rat plasma and incubated at 37°C in a capped 0.5ml tube. Samples of 2µl were withdrawn at intervals for analysis by western blotting.

Pharmacokinetic analysis of the conjugate

Male Wistar rats (of approximately 250g each) each received 20µg of ^{125}I-labeled, or 180µg of unlabeled protein in solution in phosphate buffered saline, being injected into a tail vein. Samples of blood were taken from the tail artery at intervals thereafter, with plasma prepared by heparinisation and centrifugation. Samples were analysed as described above (see Analytical Procedures) and radioactivity in whole blood was calculated as cpm/g blood. The percent injected dose (%ID) was calculated for each individual rat, based on standards and expressed as %ID/ml total blood volume. Groups of 6 or 2 rats were used for study of ^{125}I-labeled or unlabeled

protein, respectively.

Data were analysed by WinNonlin software in order to determine the pharmacokinetic values quoted. A two compartment model was used for this analysis unless otherwise stated.

Results

Characterisation of the conjugate

It is known that although serum albumin has one cysteinyl residue that is not engaged in a disulphide bond (residue 34 in the mature human albumin sequence), many of the molecules in a preparation of albumin do not possess a free cysteinyl thiol due to formation of mixed disulphides with molecules such as glutathione or free cysteine [Peters, *ibid* 1985]. In accordance with this, analysis of the rat albumin solution prior to reduction showed that there was 0.15mole of thiol per mole of albumin, i.e. only 15% of albumin molecules possessed a free thiol. After reduction, this was increased to 0.95mole of thiol per mole of albumin, consistent with presence of a free thiol group in 95% of albumin molecules, most likely at position 34 in the mature albumin sequence, since reduction under conditions such as those used here does not disrupt any of the disulphide present elsewhere in the molecule [Peters, *ibid* 1985]. Subsequent reaction at this cysteinyl residue allowed attachment of another molecule at a specific site, and in a 1:1 molar ratio, without concomitant production of other products of higher ratio of albumin:other molecule that would need to be removed subsequently. Co-ordinates for the crystal structure of human serum albumin have been deposited in the Brookhaven database (as entry 1AO6, by S. Sugio, S. Mochizuki and A. Kashima). Inspection of this model shows that the free cysteinyl residue at position 34 lies in a groove between two helices, but it is not clear from this how long a spacer arm on a crosslinking agent should be in order to work effectively. Absence of albumin homo-dimer would be advantageous in a production process since

the yield of hetero-dimer then would be greater, and also there would be no need for an additional step to remove the homo-dimer. It was found that the cross-linking agent used in the present example produced this advantageous result. Reaction of the reduced albumin with a 20-fold molar excess of BMH caused derivatisation of 80% of these albumin molecules. Albumin homo-dimers were not formed during albumin derivatisation (or subsequent reaction with other protein molecules). The cross-linking agent spacer arm, of length 16.1 Å, was long enough to reach from the cysteinyl thiol at position 34 to the surface of the albumin molecule, where it was able to react with the free thiol on a Fab' molecule. It was not long enough to penetrate the equivalent groove on a second albumin to reach the free thiol there, and so production of homo-dimers was avoided.

The derivatised albumin and Fab' were mixed in the molar ratio 1:1.4:albumin :Fab'. SDS PAGE analysis of the products at the end of reaction indicated a yield of albumin-Fab' conjugate of the order of 20 to 30%, though this would be expected to be improved by optimisation of reaction conditions. The conjugate was purified from the reaction mixture by affinity chromatography, the conjugate surprisingly not binding to the matrix as did Fab', but eluting marginally later than did albumin, which did not bind at all. Having removed unreacted Fab', albumin was separated from the conjugate by size exclusion. The final preparation of conjugate was analysed by SDS PAGE (Fig.1), which indicated (by apparent molecular weights) the linkage of one Fab' per albumin molecule. On non-reduced SDS PAGE the main band was a conjugate of 1 RSA with 1 Fab' molecule, as determined by N-terminal sequencing of the protein in the band. Approximately 37% of the conjugate was present as a higher molecular weight band that also had a RSA:Fab' ratio of 1:1. This was assumed to be dimer material, analogous to the observed occurrence of albumin polymers in untreated preparations of albumin. Lesser amounts (approximately 2% of total material) of conjugate missing L chain, and a

putative small derivative of the conjugate (approximately 4% of total) were also present. N-terminal protein sequence analysis of the main band on reducing SDS PAGE showed it to be RSA-Fd (i.e. Fab' H chain), so that the linkage was specifically via the Fab' Fd and not via the L chain. Thus the 1:1 stoichiometry of conjugation, was confirmed.

Conjugation of Fab' to cysteine (to produce the control molecule, Fab'-cys) gave good yield - virtually all the Fab' derivative reacted with cysteine with only traces of $F(ab')_2$-like material of greater than approximately 55 kDa (apparent) on non-reducing SDS PAGE. This trace of material eluted just after the Fab'-cys product, which eluted at a sodium chloride concentration of about 75mM on Mono S chromatography, and was excluded from it.

The ability to assay the conjugate in an ELISA that depended on the conjugate binding to TNF indicated that the conjugate retained the ability to bind TNF. This was confirmed by analysis of the conjugate binding to TNF by surface plasmon resonance using the BIACORE 2000 (association and dissociation rates, and equilibrium constant – see Table 1). This showed that the anti-TNF Fab' was unaffected by conjugation to albumin. This was also confirmed by its equal capacity for neutralisation of TNF: in an assay of L929 cell death caused by TNF the concentration of protein to inhibit 90% of TNF activity (the IC90) for the albumin-anti-TNF Fab' conjugate was 14.7pM, as compared to 18.0pM for anti-TNF Fab'-cysteine conjugate, 10.9pM for anti-TNF $F(ab')_2$, and 11.3pM for anti-TNF IgG4. The maintenance of Fab' binding activity was attributed to the intended orientation of the Fab' binding domain away from the point of conjugation to the albumin molecule, achieved by targeting of the Fab' hinge region as the point of attachment to the other moiety.

The control, Fab'-cys, also retained its ability to bind to, and to neutralise,

TNF, as seen by its detection by ELISA and its activity in the L929 assay.

Pharmacokinetic analysis of the conjugate in rat plasma

^{125}I-labeled RSA, Fab', F(ab')$_2$ and RSA-Fab' conjugate were monitored in plasma sampled at various times over 144h (Fig.2). The observed β-phase half-life of albumin was in agreement with the literature value of about 2 days [Peters, *ibid* 1985]. The β-phase half-life of the F(ab')$_2$, and the Fab', were similar to that of F(ab')$_2$ described previously [e.g. Kitamura *et al, ibid* ; Chapman *et al, ibid*], and was preceded by rapid elimination in the α-phase, typical of such molecules. However, when the Fab' was conjugated to RSA it persisted in circulation in the rat to a degree comparable to rat albumin. Due to a reduction of elimination during both α- and β-phases, the conjugate showed a 35-fold greater area under the plasma concentration curve (AUC, 0-∞) than did the Fab'-cys control, similar to that of albumin alone. In order to ensure that radioactivity detected in the plasma samples reflected remaining conjugate, samples from one rat given labeled conjugate were run on SDS PAGE and scanned by phosphorimager (data not shown). This showed the persistence *in vivo* of intact labeled conjugate for at least 120h, and the β-phase half-life of intact conjugate was calculated to be 32.07h, in good agreement with the equivalent result from total ^{125}I detection.

The stability of the conjugate in plasma was inspected. Incubation of the unlabelled conjugate in rat plasma (*in vitro*) at 37^{o}C for 68h in the presence or absence of a variety of protease inhibitors, monitored by SDS PAGE and western blotting, indicated that the conjugate was stable in rat plasma. Conjugate that had been labeled by ^{125}I was incubated *in vitro* in phosphate buffered saline (pH7) for up to 10 days and was also found to be stable.

However, incubation of the ^{125}I-labeled conjugate at 37°C in rat plasma or blood *in vitro* indicated that the molecule was not completely stable, some molecules suffering cleavage at or near the point of linkage between the albumin and the Fab' molecule. Since unlabeled conjugate was stable, the observed instability was attributed to modification of the protein by the presence of ^{125}I or by the labeling process. Despite an apparent instability in these conditions, intact material remained even after 168h incubation *in vitro*. The integrity of the conjugate *in vivo* was assessed by SDS PAGE of plasma samples, followed by quantification of the intact conjugate by phosphorimager scanning, and as in experiments *in vitro*, a small instability of ^{125}I-labelled conjugate was noted. The observed results from *in vivo* experiments could be adjusted accordingly to reflect the quantity of intact, labeled conjugate only. The adjusted data gave the result shown in Fig. 2 (see conjugate (correction)), which was that even the slightly unstable labeled version of the conjugate had an AUC that was 17-fold greater than the Fab'-cys control.

The conclusion that data derived from the use of ^{125}I-labeled conjugate represent an underestimate of the longevity of the unlabelled conjugate *in vivo* was supported by an experiment in which the conjugate was not labelled. Unlabeled protein was monitored by two forms of ELISA and by biological activity (TNF neutralisation in the L929 assay). Results from use of the three assays in samples from two rats were similar to each other (Fig. 3), showing that RSA-Fab' was stable *in vivo* for protracted periods. However, Fab'-cys was eliminated rapidly, such that too few data were obtained by ELISA to allow use of a two compartment model, and the AUC estimate given in Fig. 3 is by use of a one compartment model. Too few data were obtained by use of activity assay to allow any modeling at all. The RSA-Fab'conjugate's AUC was about 200-fold greater than that of the

Fab'-cys control.

It has been shown that it is possible to prepare an IgG antibody Fab' fragment, chemically crosslink it to albumin *in vitro* with retention of full binding ability (having the same affinity as seen in the whole antibody), and demonstrate that it has a significantly longer half-life *in vivo* than does the unconjugated Fab'. The conjugate contained one Fab' per albumin molecule. The β-phase half-life of the conjugate in the rat was about twice that of the unconjugated Fab, being closer to that of unconjugated albumin, and the area under the curve was about 200-fold greater for the conjugate than for the unconjugated Fab'. Thus, the consequence of conjugation of Fab' to albumin was a significant prolongation of its biological activity *in vivo*, allowing (in the present case) prolonged anti-cytokine therapy.

EXAMPLE 2. Genetic fusion of scFv to albumin.

Methods

Construction of plasmids coding for scFv-human serum albumin fusion proteins.

Plasmid pPIC(scFv) contained the gene for an anti-TNF scFv, comprising the variable domains in the order V_L-V_H. This plasmid was cleaved with restriction enzymes KpnI and NotI to generate a 'vector' DNA fragment which was purified following agarose electrophoresis. Into this was ligated a PvuII - NotI fragment of DNA containing the mature HSA gene, together with a 200bp linker encoding a direct in-frame fusion between the C-terminus of the scFv and the N-terminus of HSA. This procedure is summarised in Fig. 4. The linker or junction fragment also introduced a unique ClaI restriction site to facilitate the construction of a further series of genes encoding fusion proteins containing different spacers to physically separate the scFv and HSA motifs. Annealed oligonucleotide cassettes were used to introduce the following spacers between the KpnI and ClaI sites:

1. A sequence encoding 3 repeats of the Gly_4Ser sequence.
2. A sequence derived from the flexible N-terminal end of the human IgG1 hinge region.

In addition, a gene encoding a further fusion protein was generated in which the C-domain from human Ig-kappa light chain was used as a spacer to separate the scFv (this time in the V_H-V_L orientation) from the HSA. The construct nomenclature was: scFv-HSA; scFv-G4S-HSA; scFv-UHL-HSA; scFv-C_K-HSA, respectively.

Expression of fusion proteins in yeast.

These plasmids were linearised and transfected into competent *Pichia pastoris* cells. This was done using an "EasySelect Pichia Expression" kit (Invitrogen) by following the manufacturer's instructions. Transformants were selected by resistance to Zeomycin (500g/ml) incorporated in the YPDS agar used to grow them. Transformants were confirmed as being *Mut*$^+$, that is, capable of utilising methanol as the sole carbon source, though sensitive to the presence of excess methanol. Two colonies of each of the 4 types of transformant were picked and grown in shake flask culture in BMGY medium, as per "EasySelect Pichia Expression" kit instructions. Each culture was 25ml in a 25ml flask, incubated at 30°C, shaking at 225 rpm. After 16-18h, when the cultures had grown to give an optical density at 600nm (OD600) of between 2 and 6, cells were harvested by centrifugation and resuspended in 30 – 40ml of BMMY medium (which contains methanol as carbon source), to give an OD600 of 1. Incubation was then continued with shaking and occasional addition of methanol in order to maintain a methanol concentration of approximately 0.5% (v/v). After 84h incubation the cultures were centrifuged and the supernatants retained for use in preparation of expressed and secreted fusion proteins.

Purification of fusion proteins.

Expressed proteins were purified from the yeast culture medium after

clarification by centrifugation. The medium was subjected to chromatography on a matrix of Blue sepharose (Pharmacia), which acted as an affinity matrix for albumin-containing proteins, as follows below. Each clarified supernatant was mixed with one volume of phosphate buffered saline, pH7, and the pH of the mixture increased from about 6.5 to about 7 by addition of a small volume of sodium hydroxide solution (2M). The mixture was then applied to a 1.5ml column of Blue sepharose, pre-equilibrated in phosphate buffered saline, pH7. The flow rate at all stages was 0.5ml/min, and the temperature was 21°C. Following application, the column was washed by phosphate buffered saline until a stable baseline was achieved, and then bound protein was eluted by the eluent sodium thiocyanate, 0.2M in phosphate buffered saline. Elution was monitored by absorbtion at 280nm wavelength. Eluted protein was collected, concentrated, and its buffer exchanged to phosphate buffered saline by use of a stirred cell concentrator (Amicon, using a 10kDa nominal molecular weight cutoff filter membrane). Protein was quantified by absorbance at a wavelength of 280nm and use of theoretical absorbtion coefficient that was calculated for each fusion protein by use of the program ProtParam (found at the ExPasy website).

Radiolabeling of proteins

Fusion proteins with no linker, with G4S linker or with upper hinge linker, and unmodified human serum albumin were labelled by ^{125}I as described in Example 1, above.

Analytical procedures

Proteins were characterised and their pharmacokinetics in rats analysed as described in Example 1.

Results

Characterisation of fusion proteins

All four scFv-human serum albumin (HSA) fusion proteins were expressed from *Pichia pastoris*, being secreted into the medium at the following rates, as determined by absorbance at 280nm of protein solutions prepared by chromatography (mean of 2 experiments): scFv-HSA (no linker), 9.0μg/ml; scFv-G4S-HSA, 8.5μg/ml; scFv-UHL-HSA, 7.5 μg/ml; scFv-C_K linker, 7.8μg/ml. Bands corresponding to the expressed proteins could be seen upon SDS PAGE of unfractionated yeast culture medium (e.g. Fig. 5). No development was undertaken with the aim of improving expression levels. These data suggested that the order of the variable domains in the scFv portion did not significantly affect the levels of expression of the fusion protein.

Reducing SDS PAGE (Fig. 5) of prepared fusion proteins after only Blue sepharose chromatography showed each to contain one principle band and one minor, where the major species accounted for 91% of all protein in the scFv-HSA preparation, 75% in the scFv-G4S-HSA preparation, 80% in the scFv-UHL-HSA, and 83% in the scFv-C_K-HSA preparation, as estimated by scanning of brilliant blue G-stained gels. The principle band in each preparation had an apparent molecular weight equal to the theoretical molecular weight calculated from the translated DNA sequence. The minor band in each migrated with standard human serum albumin on the same gel, indicating minor cleavage event(s) at the sequence between albumin and scFv or scFv-hinge/spacer sequence. N-terminal sequencing of this minor product in the scFv-G4S-HSA preparation showed that it did indeed have the N-terminus of mature HSA. N-terminal sequence analysis of the fusion proteins showed them to have the expected scFv N-terminus, in each case preceded by the sequence EAEA, which had been included in the fusion protein in order to alleviate possible problem(s) of steric hindrance

rendering the cleavage of the pro-sequence inefficient (as discussed by Sreekrishna, K. *et al* (1997) Gene, 190, 55-62). This EAEA spacer sequence was not subsequently removed by yeast diaminopeptidase activity, however. Nevertheless, this N-terminal EAEA extension did not obviously inhibit expression and secretion of the heterologous proteins. It is likely that the EAEA sequence could be deleted from the fusion protein in order to generate a protein of authentic scFv (or other) N-terminus.

Blue sepharose chromatography was shown to be efficient in purification of albumin-containing proteins in a single step. These proteins included minor products of proteolysis in the present cases, but no optimisation was undertaken here. Thus, proteolysis would be expected to be reduced by such means as optimisation of culture conditions, inclusion of protease inhibitors, and/or expression in protease-deficient strains of yeast (for instance, as described by Gleeson, M. A. G. *et al* in "*Pichia* Protocols" [eds. Higgins, D. R. and Cregg, J. M.), pub. Humana Press, Totowa, New Jersey (1998), pp 81-94]. In terms of industrial processes, the cost of Blue sepharose is very low, certainly considerably lower than any other commercially-available affinity matrix. Thus, the use of albumin as part of a fusion protein is doubly beneficial, since it can not only add to the protein such properties as long serum half-life (see Example 1 or below), but also allow rapid, low cost purification. The ability of the fusion proteins to bind ligand was confirmed by Biacore analysis. This showed that the association and dissociation rates and K_D of the 4 types of fusion protein were similar to each other and to other forms of the anti-TNF antibody, including whole IgG (Table 1).

Pharmacokinetic analysis of the fusion proteins in rat plasma

The fusion proteins scFv-HSA, scFv-G4S-HSA and scFv-UHL-HSA were ^{125}I-labelled, as was HSA alone. The minor component of albumin that was present in each of the fusion protein preparations became labeled, too.

Analyses of the distribution of label in the in the fusion protein and albumin components of the preparations by the two methods of autoradiography and by phosporimaging gave similar results. The mean of the two methods was: for scFv-HSA, 76% of label was in the fusion protein; for scFv-G4S-HSA, 80% of label was in the fusion protein; for scFv-UHL-HSA, 40% of label was in the fusion protein. Remaining label was taken up by the albumin component of each preparation.

These proteins were injected into rats (n=6). Plasma samples were taken at intervals for analysis by gamma counting and by SDS PAGE followed by phosphorimaging. The distribution of label in the different samples were determined during the first 48h by phosphorimaging of samples from one rat per group (replicate number, n=2 or 4). In each case there was found to be no clear change in the distribution of label during the 48h period (data not shown). Therefore, the fusion proteins were not prone to significant degradation *in vivo*. It was assumed that this stability was maintained for the full duration of the experiment, which was 144h, and the pharmacokinetic analysis of the proteins was based on the results of gammacounting. The pharmacokinetics were similar for all proteins (see Fig. 6), thereby showing that the fusion proteins behaved similarly to unmodified human albumin in plasma *in vivo*. Note that in rats HSA is only a half (or less) as persistent as the homologous, rat, albumin, and a fusion of scFv with HSA would not necessarily be expected to endow a half-life any longer than that of HSA itself. Nevertheless, the AUC for the ^{125}I-fusion proteins are approximately 13-fold greater than those for ^{125}I-Fab'-cys control (shown in Fig. 2).

This example shows that serum albumin may be fused to another protein(s) to generate a fusion that possesses function(s) of the other protein(s) together with the long half life of albumin. Furthermore, since fusion did not involve albumin cysteinyl thiols, the possibility remains of using a fusion

protein as an acceptor of one or more Fab' molecules, linked by chemical means, as exemplified by Examples 1 and 3. Polyspecific as well as polyvalent molecules could be generated in these ways.

Example 3.

Fab' dimer-albumin conjugate.

Methods.

Preparation of the dimer of anti-cell surface marker Fab'.

The anti-cell surface marker was engineered as a Fab'. It was expressed in *E. coli* and was extracted from the periplasm, as described in International Patent Specification No. WO98/25971.

The Fab' interchain disulphide bond was reduced as follows: 12ml of Fab' at 20.93mg/ml in 0.1M sodium phosphate, pH6.0, 2mM EDTA was mixed with 240μL 250mM 2-mercaptoethylamine in the same buffer, and incubated for 50 min at 38°C. Diafiltration of the sample against the same buffer removed the reducing agent. The yield of Fab' at this stage was 85.1%. Thiol assay showed there to be an average of 0.83 free thiols per Fab' molecule, and analysis by GF250 size exclusion chromatography indicated the protein to comprise 93.1 Fab' monomer and 6.9% disulphide-bonded $F(ab')_2$.

The crosslinking agent was a trimaleimide compound, illustrated in Fig. 7. This molecule also contained a metal-chelating function. A 1mg/ml solution of this compound was made in 0.1M sodium phosphate, pH6.0, 2mM EDTA, and 5 aliquots of 434.8 μl each added with mixing at 5min intervals to the above reduced Fab' preparation. This provided a molar ratio crosslinker:Fab':1:2.56. The mixture was incubated at 21°C for 3h, at which time 25μl glacial acetic acid was added to lower the pH to 4.5. 47ml water was added to lower conductivity and the mixture resolved by ion exchange chromatography. The proteins were applied to a 2.6cm i.d. x 15.5cm column (Pharmacia) containing methyl sulphonate cation exchanger (SP-

Sepharose HP) at a flow rate of 10.5ml/min, and eluted by a gradient of 0 to 250mM sodium chloride in 50mM acetate, pH4.5.Elution was monitored by absorbance at 280nm. The various reaction products (monomer, dimer, and trimer) were identified by SDS PAGE and by GF250 size exclusion chromatography. Only that fraction containing dimer Fab' was used for the subsequent conjugation to albumin.

Linkage of Fab' dimer to rat serum albumin.

33.71mg of RSA was dissolved in 2.5ml of 0.1M sodium phosphate, pH6.0, 2mM EDTA and mixed with 25µL of 200mM 2-mercaptoethylamine in the same buffer, giving a final reducing agent concentration of 2mM. The mixture was incubated at 37°C for 1h. Reducing agent was then removed by size exclusion chromatography on Sephadex G25M, using a PD10 column (Pharmacia, code no. 17-0851-01, used as per manufacturer's instructions. The buffer was then sodium phosphate, 0.1M, pH6, 2mM (EDTA). The concentration of albumin was 143µM.

The reduced albumin was incubated with conjugated Fab' dimer in a molar ratio of 1:1, and incubated at 21°C for 24h. The reaction mixture was resolved by chromatography on Gammabind plus, as described in example 1 (above), using a column of 3ml volume. Unreacted albumin failed to bind to this matrix. Bound material was eluted by a buffer of 0.5M acetic acid, pH3. Eluted fractions were immediately neutralised by addition of trizma, 2M, pH 8.8. This material was further purified by Blue sepharose chromatography, as described in Example 2. Unreacted Fab' dimer failed to bind to the matrix, and bound material was eluted by sodium thiocyanate, 0.2M in phosphate buffered saline. Eluted fractions were concentrated and the buffer exchanged for phosphate buffered saline in a stirred cell (Amicon) with a 10kDa nominal molecular weight cutoff filter. Each type of chromatography was repeated in order to completely purify the albumin-Fab' dimer conjugate.

Analytical procedures.

Procedures were as described in Examples 1 and 2, except that for N-terminal protein sequence determination, the automated sequencer used was a PE Biosystems Procise 492. Additionally, the ligand binding characteristics of the conjugate were determined by Scatchard analysis of its binding to whole cells, as follows below.

The conjugate and a control protein (a dimer of Fab' crosslinked through thiols by bis-maleimido hexane) were labelled by attachment of fluorescein. This was done by incubation of one mg protein in 1ml of 0.1M $NaHCO_3$ with 100μg fluorescein isothiocyanate (Sigma F7250) added as 10μl solution in dimethyl sulphoxide. Reaction was for 2h at 21°C. The reaction was stopped by addition of a molar excess of lysine. Labelled protein was separated from free fluorescein by size exclusion chromatography. The fluorescein-labelled antibody was collected in phosphate-buffered saline, and the protein component quantified by absorption at 280nm, while the extent of substitution was estimated from absorption at 495nm. The μM extinction coefficient used for DFM was 0.14, for RSA-$(Fab')_2$ was 0.2 and for fluorescein was 0.077. The fluorescein-labelled control protein had a fluorescein:protein molar ratio of 1.59, and the fluorescein-labelled RSA-$(Fab')_2$ had 3.08 fluorescein molecules per protein molecule.

The fluorescent protein conjugates were then serially diluted (1:1.4) from a top concentration of 2μg/ml in phosphate buffered saline containing 5% (v/v) foetal calf serum. The final volume per tube was 250μl. Forty thousand target cells were added in 100μl per tube to give a final volume of 350μl and a final highest antibody concentration of 1.43μg/ml. Cells were incubated with antibody at 4°C for 3h. Fluorescence activated cell sorting (FACS) analysis was then performed on the cells. Fluorescence signal was converted to molecules of equivalent soluble fluorescein from a standard curve of fluorescent beads according to the method of Krause *et al.*, (1990)

Determination of Affinities of Murine and Chimeric Anti-α/β-T Cell Receptor Antibodies by Flow Cytometry. Behring Inst. Mitt. 87,56-67). The number of molecules of fluorescein bound per cell was converted to the number of molecules of antibody bound per cell by the fluorescein:protein ratio. Subtraction from the total number of antibody molecules per tube gave the number of molecules free per tube. Scatchard analysis was performed on these data.

Results.

In the non-optimised conditions used, the yields of products from the chemical crosslinking reaction were: Fab' trimer, 30.6%; Fab' dimer, 30.7%; Fab' monomer, 38.7%.

This dimer was used in subsequent reactions with albumin. Being produced by crosslinking with a tri-functional agent, the dimer possessed one functional group remaining on the linking moiety. This was a maleimide that was able to react with a free thiol. A free thiol was generated in rat albumin by reduction, by a method that was different from that used in Example 1. Assay of thiols in the albumin preparation reduced in this way showed that there was then an average of 0.97 free thiols per molecule. Thus, a single thiol may be generated in albumin by more than one method.

Incubation of the prepared Fab' dimer with the reduced RSA gave a yield of 1mg of albumin-Fab' dimer conjugate, as estimated by absorbance at 280nm, using a theoretical absorption coefficient calculated by the program ProtParam (ExPasy), after complete purification.

SDS PAGE (reduced) of the product showed the presence of two bands, of apparent molecular weight of approximately 150kDa and approximately 31kDa (Fig. 8). N-terminal sequencing showed these to be albumin-Fab' heavy chain dimer, and Fab' light chain, respectively. Non-reduced SDS

PAGE showed one main band of apparent molecular weight about 200kDa. N-terminal protein sequencing showed this band to be albumin-Fab' (i.e. heavy plus light chains) dimer. That the product of combination of RSA and Fab' dimer consisted of one albumin molecule and two Fab' molecules was confirmed by N-terminal sequencing of unfractionated final product, also. This also showed that no Fab' light chain had dissociated during production and that the covalent linkage between Fab' and albumin was via the Fab' heavy chain, only.

Ligand binding activity was retained by the conjugate, as shown by FACS analysis of its binding to cells bearing ligand on their surface. Scatchard analysis of binding gave a 2-phase curve, with a K_D of 8nM for binding of one of the two Fab's, and 3nM for binding of both Fab's in the construct (Fig. 9). These values were similar to those of a control (Fab' dimerised by crosslinking by bis-maleimido hexane i.e. lacking the albumin moiety), being 12nM and 3nM, respectively. As in the other two Examples, maintenance of the Fab's full binding activity despite the attachment of a large molecular weight moiety was probably due to the targeting of the Fab' hinge as the point of attachment, being at the opposite end of the molecule from the antigen binding function. In the present Example, the albumin interfered with neither Fab' to which it was attached.

Thus, this process produced a very specific, bivalent product. In contrast to Example 1, this was by modification of the immunoglobulin moiety, rather than the albumin, prior to the final stage of conjugation. Again in contrast to the other examples, a different immunoglobulin was used, exemplifying the general utility of the approach. Using such approaches, linkage of albumin to other polyspecific immunoglobulins would clearly generate polyspecific molecules with extended half-life *in vivo*.

The present results also exemplify inclusion of extra function in the

conjugate. The present example is inclusion of a metal-chelating function, such as might be useful for assay, diagnosis or therapy. The chelating function in this example is on the linking moiety, and is a macrocycle that has previously been found to strongly bind metals, notably Indium, but possibly alternatively Copper, Gadolinium, Iron III, Cobalt III, Chromium III, Nickel or Aluminium.

TABLE 1.

Surface plasmon resonance analysis of binding of ligand to conjugate, fusions and other immunoglobulins.

All immunoglobulins and derivatives were derived from the same anti-TNF antibody, and TNF was used as the ligand. See Figure 10 for analysis of binding of RSA-F(ab')$_2$.

Table 1: Surface plasmon resonance analysis of binding of ligand to conjugate, fusions and other immunoglobulins

	k_a, 10^5 $M^{-1}s^{-1}$	k_d, 10^{-4} s^{-1}	K_D, 10^{-10} M
IgG	3.63	1.41	3.88
Fab'	2.79	0.56	2.01
RSA – Fab' conjugate.	3.88	1.65	4.25
scFv-HSA	7.67	1.11	1.45
scFv-G4S-HSA	6.58	1.32	2.29
scFv-UHL-HSA	6.64	1.52	2.29
scFv-C_K-HSA	7.09	0.95	1.34

CLAIMS

1. A multi-component hybrid protein comprising one or more antigen-binding antibody fragments covalently linked to one or more serum carrier proteins or fragments thereof.

2. A hybrid protein according to Claim 1 comprising one, two or three antigen-binding fragments covalently linked to one serum carrier protein or a fragment thereof.

3. A hybrid protein according to Claim 1 or Claim 2 wherein each serum carrier protein is thyroxine-binding protein, transthyretin, α1-acid glycoprotein, transferrin, fibrinogen or albumin or a fragment thereof.

4. A hybrid protein according to Claim 1 to Claim 3 wherein each antibody fragment is a monovalent Fab fragment optionally containing one or more additional amino acids attached to the C-terminus of the CH1 domain.

5. A hybrid protein according to Claim 4 wherein the antibody fragment is a monovalent Fab or Fab' fragment.

6. A hybrid protein according to any one of the preceding claims comprising one antigen-binding antibody fragment covalently linked to an albumin molecule or a fragment thereof.

7. A hybrid protein according to Claim 6 wherein the antibody fragment and albumin are directly linked through the C-terminal amino acid of the antibody to the N-terminal amino acid of the albumin optionally through a spacing group.

8. A hybrid protein according to Claim 6 wherein the antibody fragment and albumin are indirectly linked by a bridging molecule between the thiol groups of a cysteine residue present in the antibody and another in the albumin.

9. A hybrid protein according to Claim 8 wherein the cysteine residue present in the albumin is at position 34.

10. A hybrid protein according to Claim 8 or Claim 9 wherein the bridging molecule is from around 10Å to around 20Å in length.

11. A hybrid protein according to Claim 10 wherein the bridging molecule is an optionally substituted hexylene chain.

12. A hybrid protein according to any one of the preceding claims covalently linked to one or more effector or reporter groups.

13. A pharmaceutical composition comprising a hybrid protein according to any one of the preceding claims together with one or more pharmaceutically acceptable excipients, diluents or carriers.

FIG. 1 SDS PAGE of RSA-FAB' conjugate

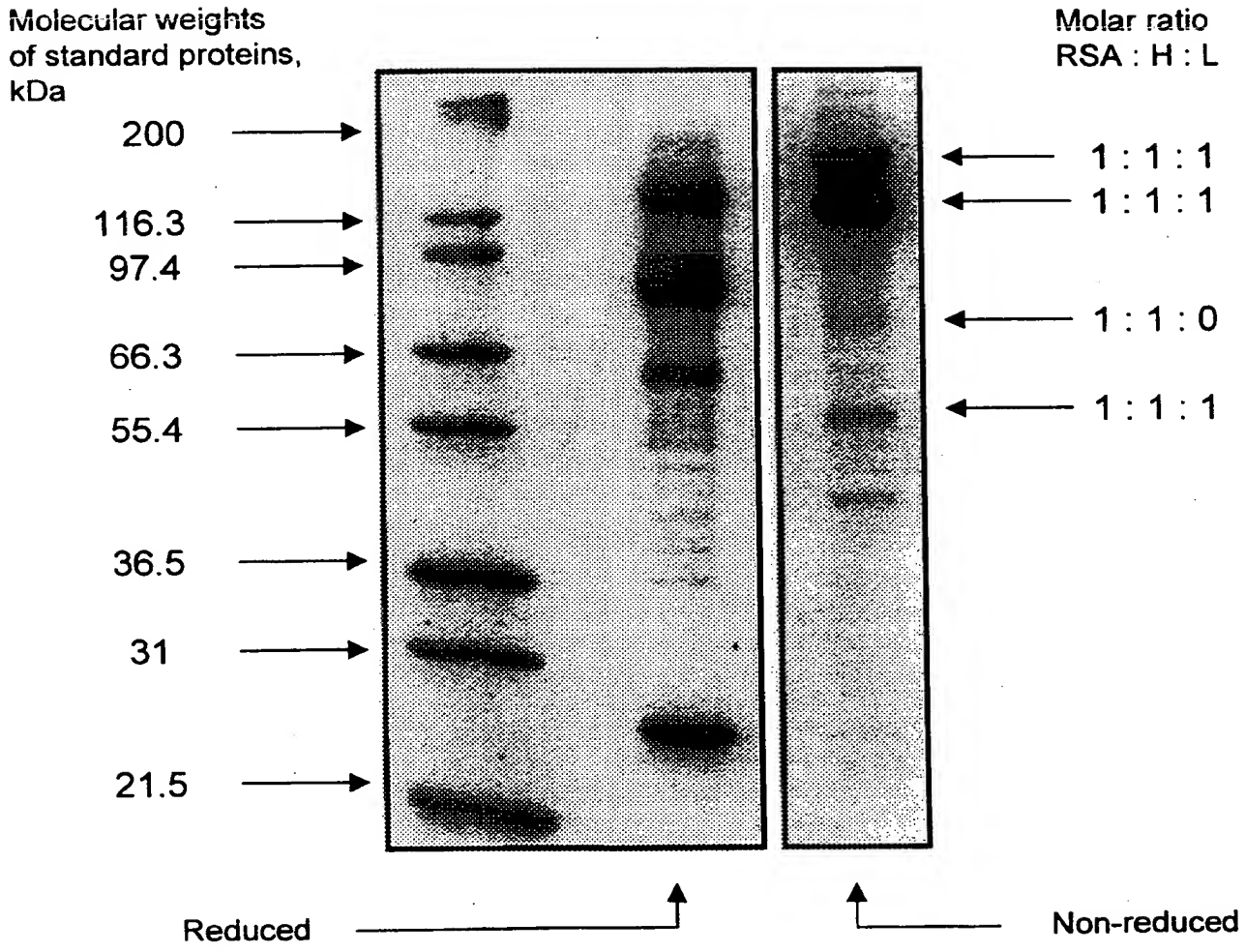


FIG. 2 Pharmacokinetics of RSA-Fab' conjugate or controls in the rat

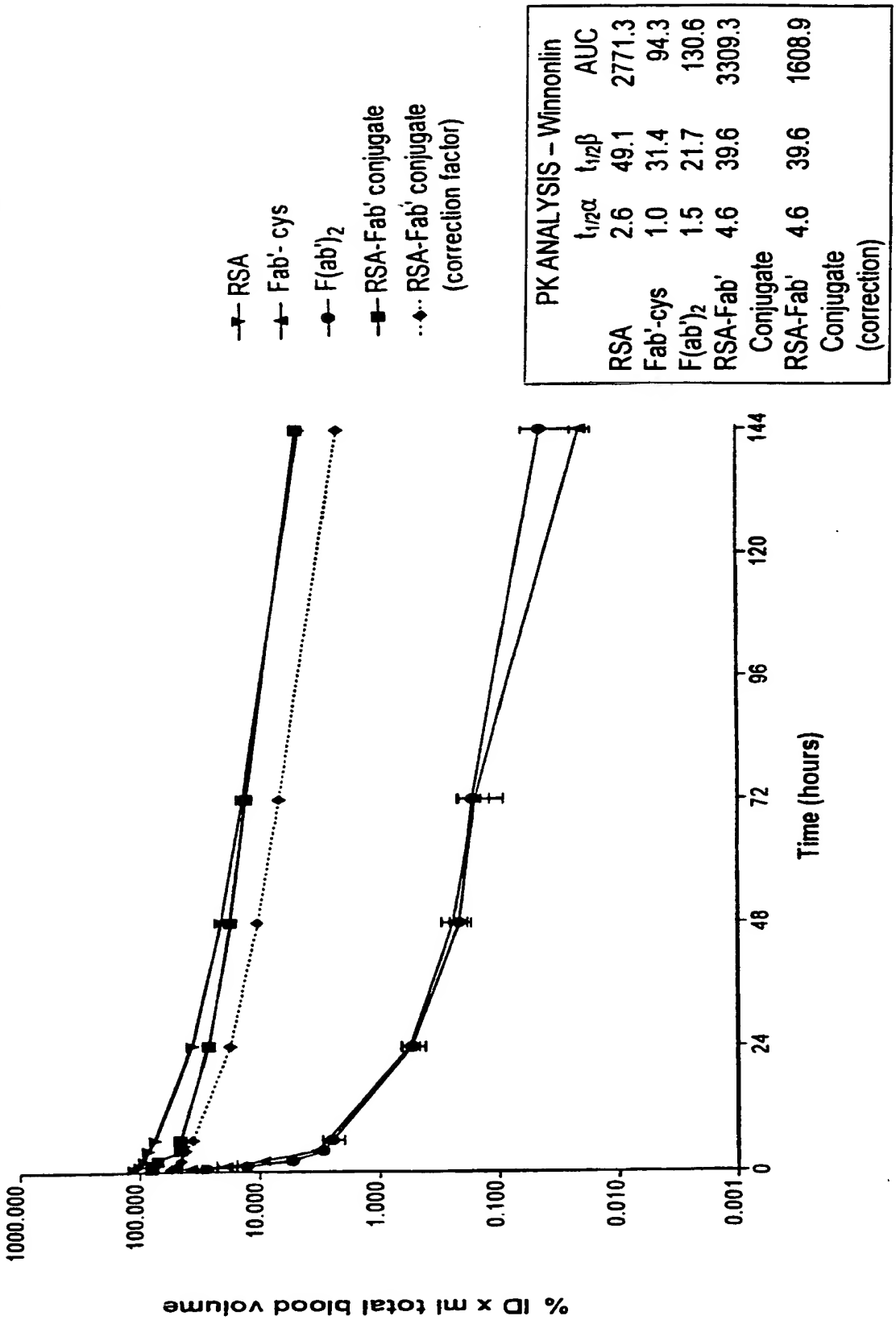


FIG. 3 Pharmacokinetics of RSA-Fab' or Fab'-cys in the rat (assayed by ELISA or cytokine neutralisation

PK ANALYSIS - Winnonlin	$t_{1/2}\alpha$(h)	$t_{1/2}\beta$(h)	AUC (0-∞) (h*µg/ml)
L929 assay:			
RSA-Fab' conjugate	2.7	24.7	975
Fab'-cys	ND	ND	ND
ELISA assay:			
RSA-Fab' conjugate (ligand-albumin)	0.8	19.5	780
RSA-Fab' conjugate (ligand-L chain)	1.3	21.6	824
Fab'-cys	ND	0.7	4

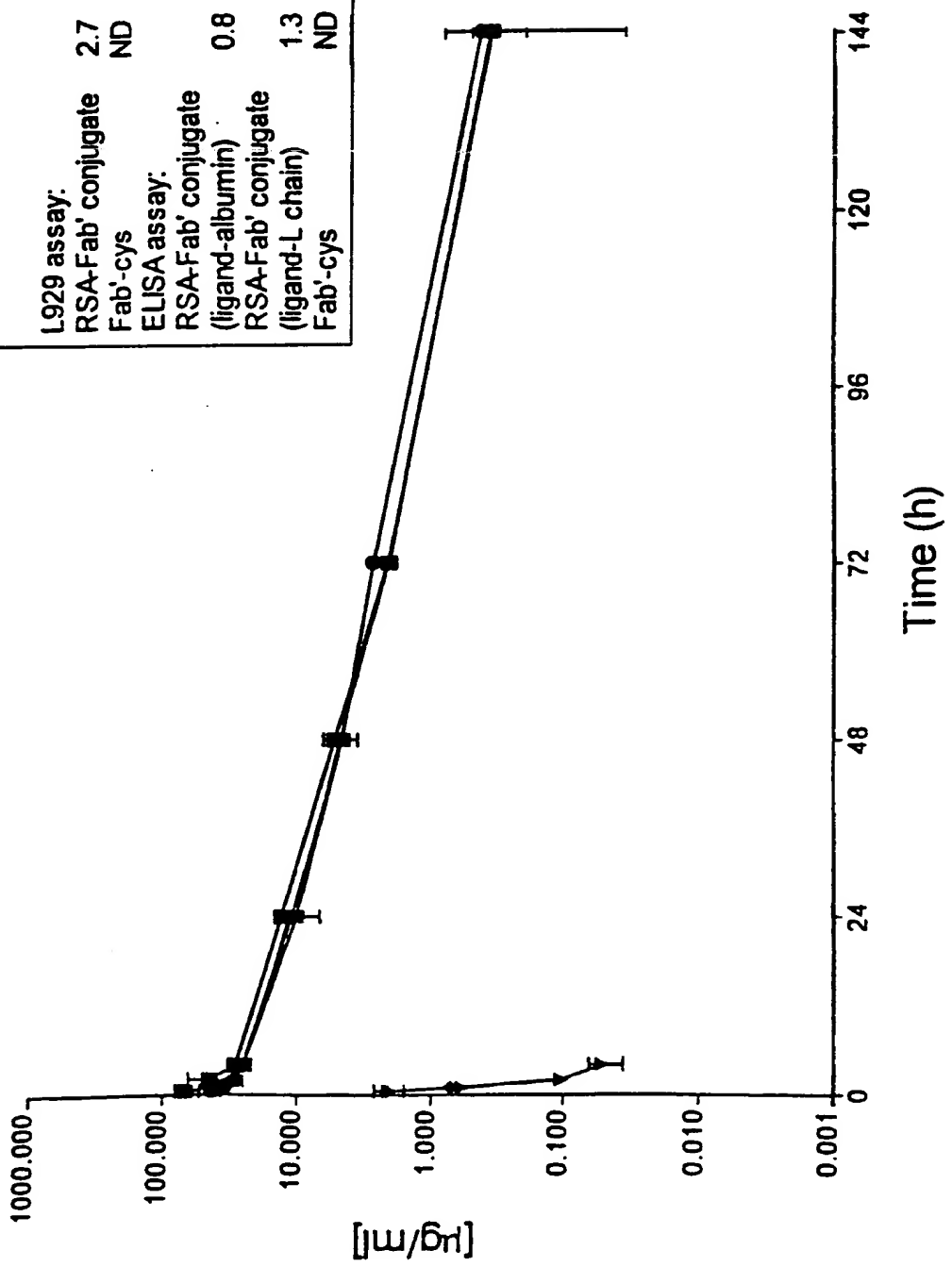


FIG. 4 Construction of pPIC (scFv-HSA)

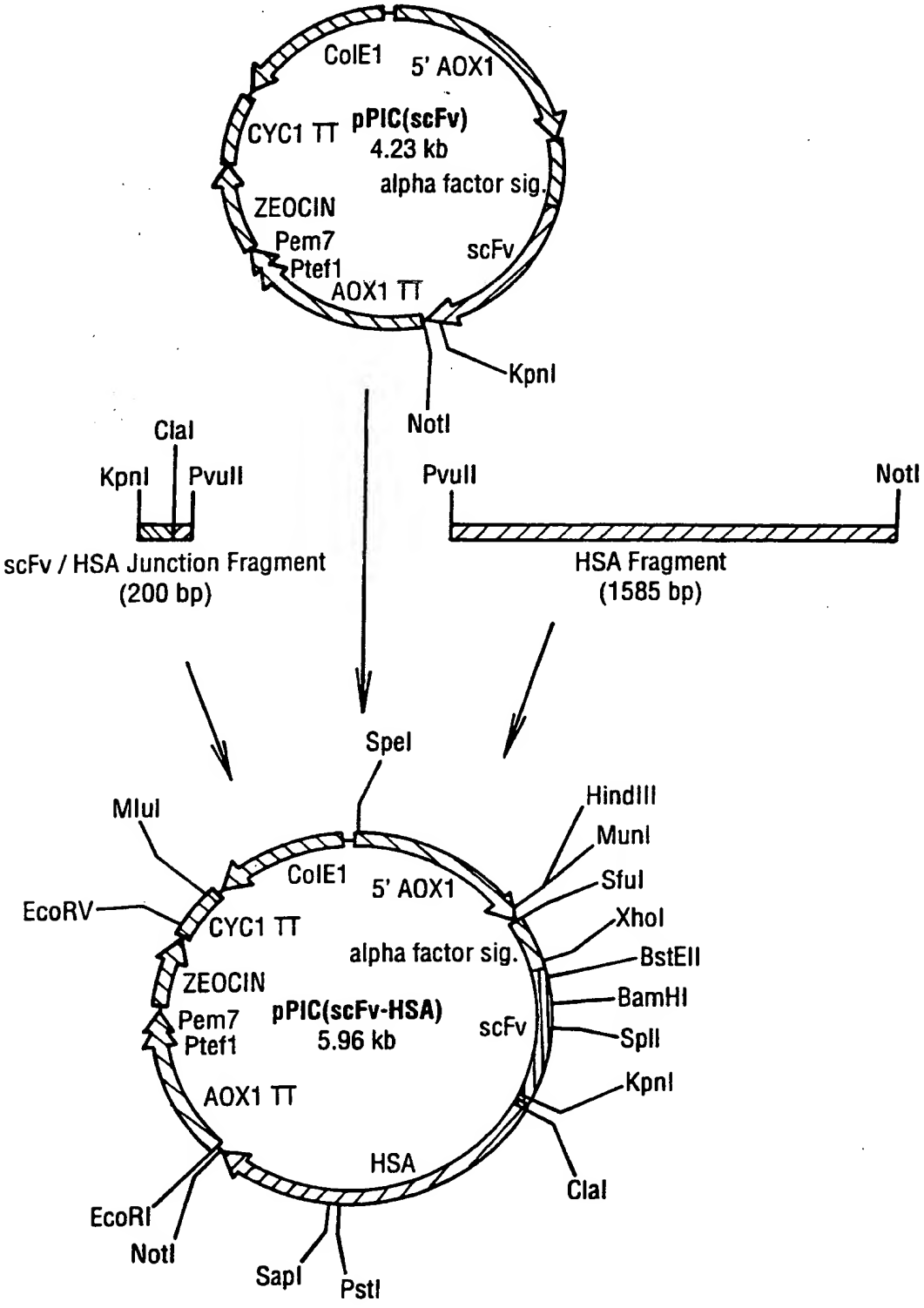


FIG. 5 Reducing SDS PAGE of HSA-scFv fusion proteins

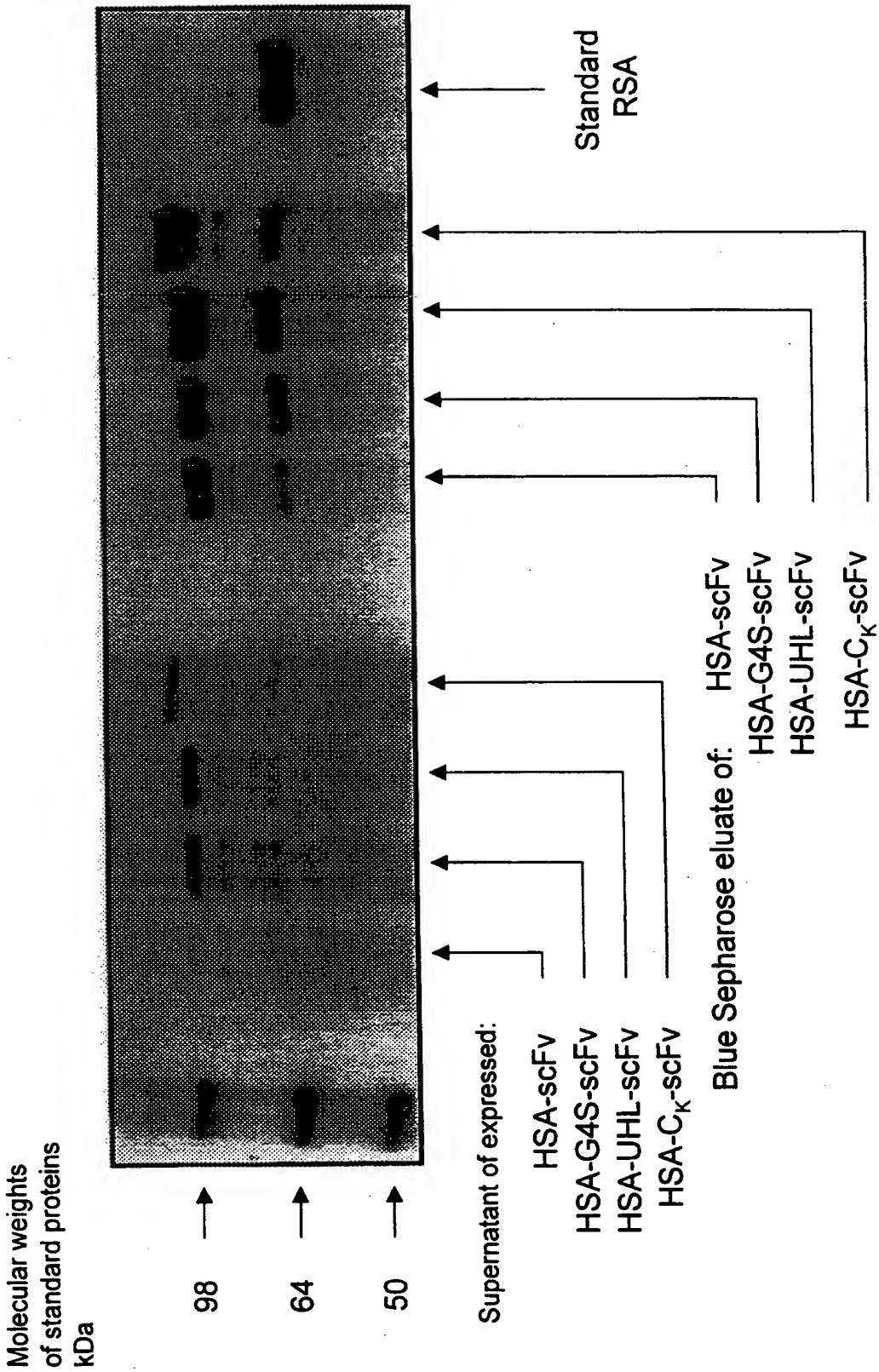
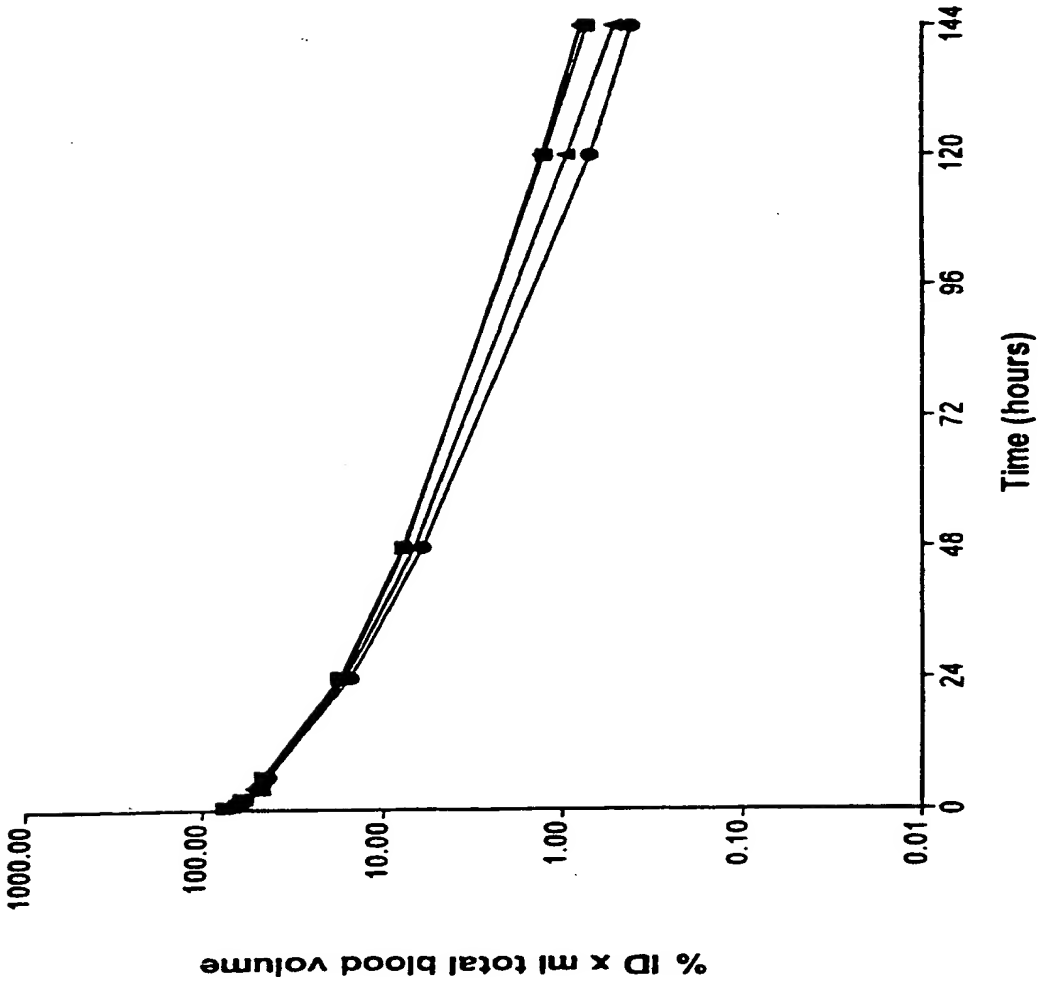


FIG. 6Pharmacokinetics fo HSA-Fab' fusion proteins or HSA in the rat



PK ANALYSIS	$t_{1/2}\alpha$(h)	$t_{1/2}\beta$ (h)	AUC(0-∞) (h*%dose)
ScFv-HSA	2.28	16.63	1163.83
ScFv-G_4S-HSA	0.98	15.21	1281.97
ScFv-UHL-HSA	3.02	16.48	1224.78
HSA	0.77	14.77	1271.6

FIG. 7 Structure of trimaleimide crosslinking agent

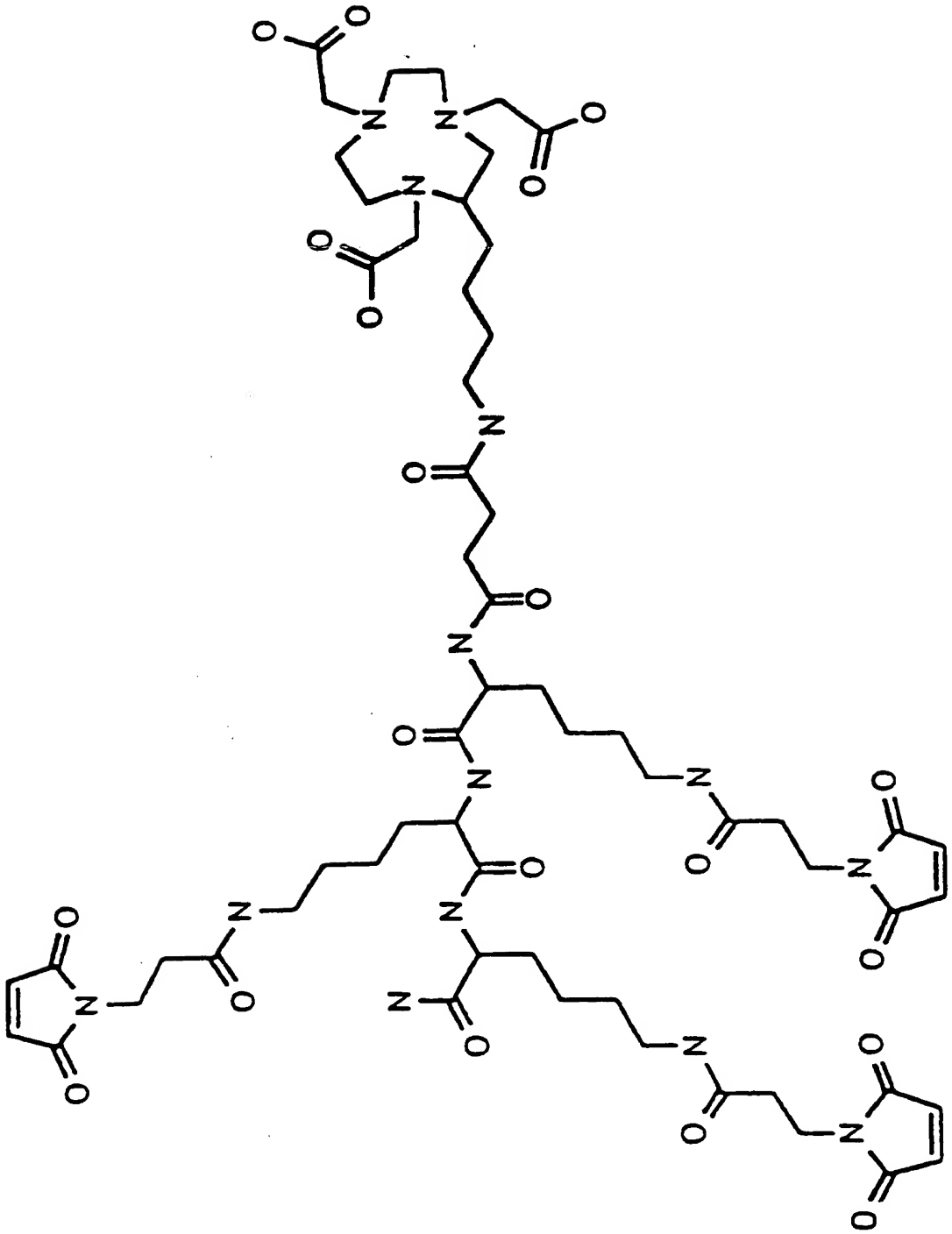


FIG. 8 Reducing SDS PAGE of HSA-F(ab')$_2$ conjugate

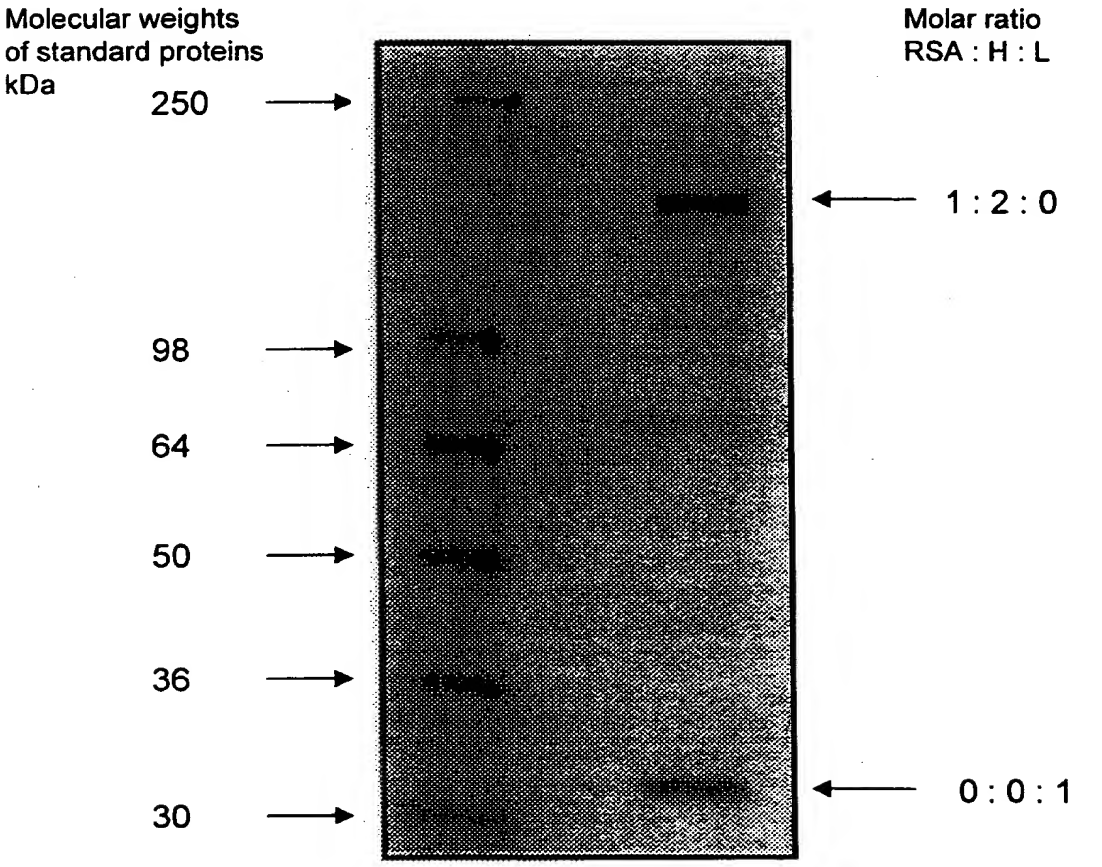
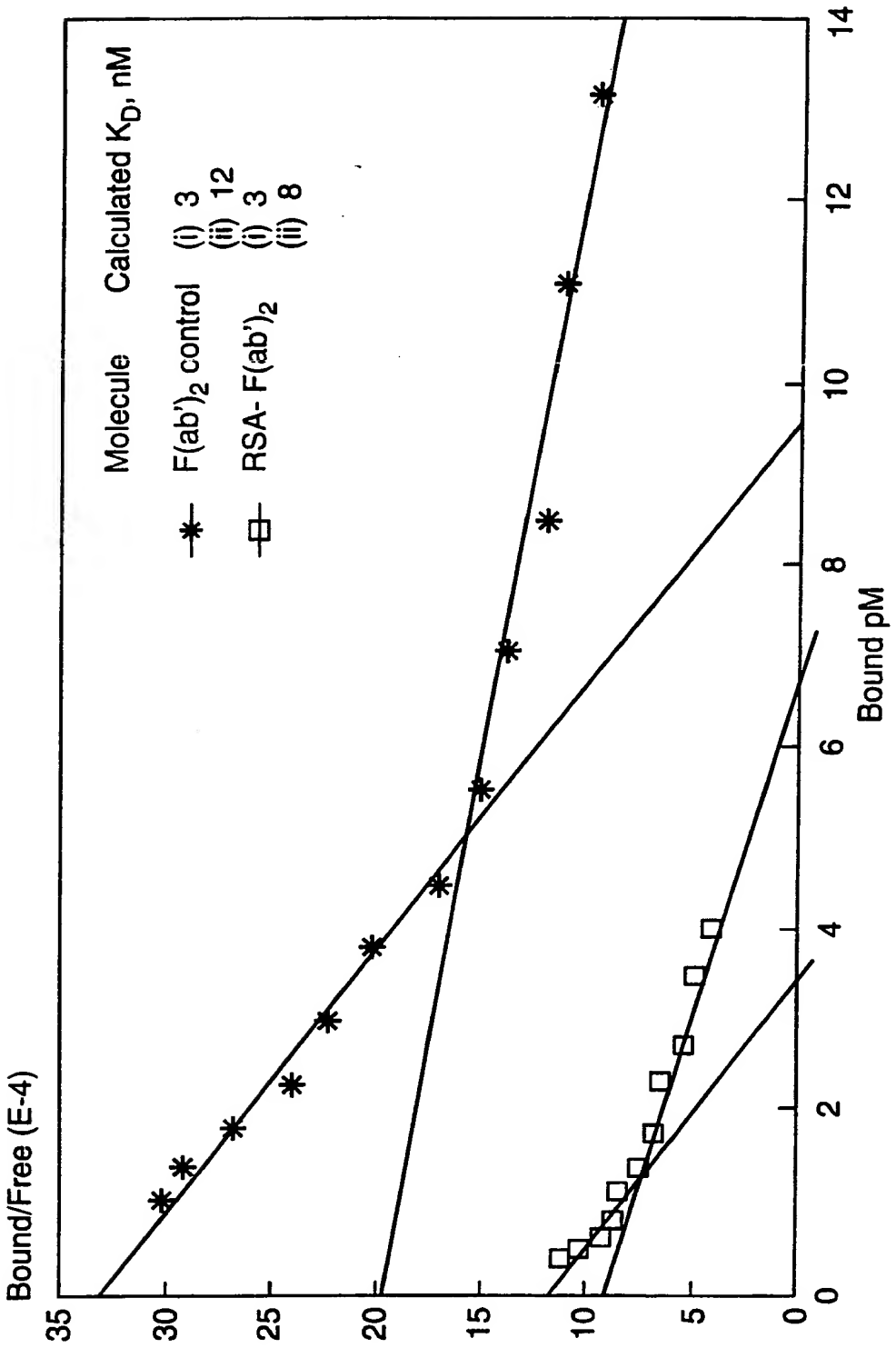


FIG. 9 Scatchard plots of binding of RSA-F(ab')$_2$ or F(ab')$_2$ control to cell membranes



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 99/03747

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 00763 A (AKZO N. V., NETH.) 23 January 1992 (1992-01-23) page 2, paragraph 3 -page 3, paragraph 3 page 4, paragraph 2 example 2	1-13
X	WO 95 33492 A (HOUSTON BIOTECHNOLOGY) 14 December 1995 (1995-12-14) figure 1 page 8, line 13-24	1-13

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 February 2000	16/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Covone, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible]nal Application No
PCT/GB 99/03747

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9200763 A	23-01-1992	EP 0537222 A	21-04-1993
		FI 925804 A	21-12-1992
		HU 63343 A	30-08-1993
		NO 924762 A	29-12-1992
WO 9533492 A	14-12-1995	AU 2823495 A	04-01-1996
WO 9734631 A	25-09-1997	AU 1985097 A	10-10-1997
		CA 2249195 A	25-09-1997
		EP 0904107 A	31-03-1999